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THE SELECTIVE ACTION OF NICOTINE ON THE CENTRAL NERVOUS SYSTEM OF THE SQUID, LOLIGO PEALII.

By A. R. MOORE.

(From the Physiological Laboratory of Rutgers College, New Brunswick, N. J., and the Marine Biological Laboratory, Woods Hole, Mass.)

(Received for publication, February 19, 1919.)

Juvenile squid, *Loligo pealii*, are excellent indicators of the action of neurophil alkaloids. Excitation is shown by spasms of the musculature, in tentacles and mantle, and expansion of the chromatophores. This latter effect is caused by contraction of the radial muscle fibers attached to the sac-like chromatophores. The central nervous system of the squid is formed by the large cerebral ganglion to which are connected by nerve strands the two mantle or stellar ganglia. The musculature of the mantle may be excited either by the activity of the stellar ganglia or by that of the cerebrum. By means of observations on decapitated specimens it is thus possible to determine the seat of the nervous excitation. Strychnine in concentrations as low as 1: 500,000 causes the entire musculature to show spasms, with accompanying extension of the chromatophores in tentacles, head, and mantle. Since this occurs in decapitated as well as in entire specimens, it must be concluded that strychnine acts upon both groups of ganglia.[1] Camphor acts in a similar way, causing mantle spasms in decapitated animals, the excitatory action of camphor on the stellar ganglia being thereby proven.[2]

Nicotine is said to have an action in vertebrates similar to that of other excitants of the central nervous system.[3] With respect to its central action, this alkaloid falls in Maxwell's second group of brain stimulants since it acts upon ganglia alone.[4] In view of this selective

[1] Baglioni, S., *Z. allg. Physiol.*, 1905, v, 43.

[2] Moore, A. R., *Proc. Nat. Acad. Sc.*, 1917, iii, 598.

[3] Meyer, H., and Gottlieb, R., Pharmacology, Philadelphia, 1914, 373.

[4] Maxwell, S. S., *Am. J. Physiol.*, 1918, xlvii, 283; *J. Biol. Chem.*, 1907, iii, 21.

action, it seemed of interest to determine further whether nicotine is equally active on the stellar and cerebral ganglia of squid. For this purpose the freshly hatched animals were immersed in sea water solutions of Kahlbaum's alkaloidal nicotine. Typical effects were observed with dilutions as great as 1: 5,000,000, but stronger concentrations were found to be more satisfactory since they gave prompter results. It was noted also that ordinary commercial preparations of nicotine gave results identical with those of the purified alkaloid.

Upon being put into a solution of nicotine in sea water, the squid, in 3 or 4 seconds, falls to the bottom of the dish, with mantle shortened and broadened, quiescent, chromatophores relaxed, but with head drawn in and chromatophores of head and tentacles extended. After several minutes a secondary effect appears. If the dish is tapped, head and mantle are extended, accompanied by muscular spasms and maximum play of chromatophores over the entire body, while ink is often shot.

In order to determine whether the action of nicotine in producing such mantle spasms is due to its action on the head or mantle ganglia or both, experiments were carried out with decapitated mantles. These showed neither spasms nor play of the chromatophores, whether the decapitation took place before or after placing them in the nicotine solution. In order to remove any doubt in the matter, animals were kept in the solution of the alkaloid until the mantle spasms had developed, and were then decapitated, with the result that the mantles immediately relaxed and the chromatophores closed. Since in control experiments, in which decapitated mantles were immersed in sea water containing strychnine or camphor, spasms and chromatophore extension occurred, it is necessary to conclude that nicotine produces mantle spasms by acting upon the cerebral ganglion alone.

In a sea water solution of nicotine 1: 500,000 the mantle spasms develop after 6 to 7 minutes at 24°C. It made no difference in the time required for the onset of this effect whether the animals were removed from the solution and put into the sea water at the end of 1, 2, 3, 4, or 5 minutes or simply remained in the nicotine solution; the spasms in all cases appeared promptly 6 or 7 minutes after immersion in the poison. This somewhat astonishing result may be

accounted for if we assume it to be due to the formation of a compound between the nicotine and some substance of the cerebrum in critical concentration, and that sufficient nicotine is taken into the squid's body during the first minute to accomplish this result, and further that the uncombined nicotine is retained during the 6 or 7 minutes necessary for the reaction to reach the desired point. The assumption that this reaction or series of reactions is essentially chemical in nature is supported by the effect of the temperature on the length of the latent period.

Temperature. C.	Time. min.	Temperature coefficient.
24°	7	
14°	20	2.86
24°	6	
14°	17	2.83
23°	8	
13°	22	2.75

The rate of the reaction is increased approximately 2.8 times for an increase in temperature of 10°C. This value falls in well with the temperature coefficients of other life phenomena.

The effect of the concentration of the nicotine on the latent period of the mantle spasm is shown below. $T° = 24°$. t = time in minutes. v = the velocity of the process.

Concentration of nicotine.	Concentration ratio.	t	$v = 16.2 \times \frac{1}{t}$	$\sqrt[3]{\text{concentration}}$
1: 50,000	100	3	5.4	4.75
1: 500,000	10	7	2.3	2.2
1: 1,000,000	5	11	1.5	1.7
1: 5,000,000	1	14	1.1	1.0

From these results it appears that the velocity of the process varies approximately as the cube root of the concentration of the nicotine.

SUMMARY.

1. In specimens of freshly hatched squid, *Loligo pealii*, nicotine acts upon the cerebral ganglia alone.

2. After 1 minute in the nicotine solution 1: 500,000, the latent period for the mantle spasm is independent of the time spent in the solution.

3. The mantle spasm is conditioned by a chemical reaction, since the temperature coefficient of the process has a magnitude of about 2.8.

4. The velocity of the process which brings about the mantle spasm varies as the cube root of the concentration of the nicotine.

ON THE EXPERIMENTAL PRODUCTION OF EDEMA BY NEPHRECTOMY.

By W. W. SWINGLE.

(*From the Laboratory of Comparative Anatomy, Princeton University, Princeton.*)

(Received for publication, March 27, 1919.)

The question of the causation of edema is one that has interested clinical pathologists and other investigators for many years. Though much has been done to clear up certain phases of the problem, it has still remained in doubt to what extent, if any, a generalized edema of the body may be due to an inefficiency of the kidney function. Recently, however, the work of McClure[1] has shed considerable light upon this obscure problem.

McClure has shown that the edematous condition frequently met with in young frog larvæ is invariably associated with a deficiency in the development of the head kidney (pronephros). Furthermore, he has been able to produce an edematous state of the body at will in adult frogs and toads by ligation of the ureters. His experiments clearly show that a generalized edema of the body may result in Anura from a block in the kidney function.

The writer concluded that it might be of interest in this connection to extirpate the pronephros or head kidney of very young frog larvæ, shortly before the period when this organ becomes functional. According to the viewpoint of McClure edema should result in such cases. The results justified expectations, as the following experiments show.

Several egg masses of *Rana sylvatica* LeConte were collected and brought to the laboratory to develop. Evidently the eggs had been laid early in the morning of the preceding day, for most of them were in early segmentation stages (8 to 16 cells). The larvæ hatched within a few days and were apparently normal in every respect.

[1] McClure, C. F. W., On the experimental production of edema in larval and adult Anura, *J. Gen. Physiol.*, 1918–19, i, 261.

Larvæ of 5.5 to 7 mm. in total length are in the proper developmental stage for successful extirpation of the pronephros and Wolffian ducts. Slightly older larvæ (7 to 8 mm.) give fair results but are not so easily handled as the younger stages. All extirpations were performed under the binocular microscope with a blunt dissecting needle and a razor-edged, spear-head needle. The larvæ were narcotized with chloretone.

The glandular portion of the pronephros in larvæ of 5.5 to 7 mm. in length shows very clearly as a small rounded elevation or nodule, just caudal to the gills, on either side of the head. This elevation represents the position of the coiled pronephric tubule[2] with its three nephrostomes which open into the cœlomic cavity. The Wolffian ducts extend caudad and lie just ventral and medial to the edge of the heavy band of musculature or myotomes. In favorable specimens, with good illumination, the exact location of the Wolffian duct is represented by a very faint line in the position just described. The ducts in the later stages open dorsally into the cloaca. The operation of removing the pronephros or its duct is simple and the wound closes within an hour or two.

Total Nephrectomy.

The glandular portion of the pronephros was removed on both sides of the body from 110 larvæ. In many of the smaller larvæ of the group, *i.e.* in those measuring 5.5 mm., it was observed that the Wolffian ducts had not yet opened to the exterior. The larvæ, appropriately controlled, were left in well aerated water over night. On the following morning, examination showed all the larger 7 mm. larvæ to be edematous. The edema in its incipient stages appeared chiefly as a swelling of the submaxillary lymph sinus.

20 hours after the operation, 16 larvæ showed marked edema. 5 hours later 36 were edematous; at the end of 36 hours, 97 larvæ showed a typical edema. The few larvæ which remained normal

[2] Field, H. M., The development of the pronephros and segmental duct in amphibia, *Bull. Mus. Comp. Zool.*, 1891, xxi, 201. Huber, G. C., On the morphology of the renal tubules of vertebrates, *Anat. Rec.*, 1917, xiii, 305.

showed upon microscopic examination a turgor of the lymph spaces, and finally after several hours more (the longest 48) all larvæ were edematous. The mortality was very slight up to 36 hours. After this time, the extremely edematous larvæ usually burst, unless killed and preserved in fixing fluids. Rupture of the body wall usually occurred at the point of operation.

Several greatly swollen larvæ were observed to burst while being examined. These were at once placed in separate containers and carefully watched. Several hours later these same larvæ were again observed to be edematous. They were then left over night. Two were found dead the next morning. The remaining two had burst again during the night; they recovered but again became edematous and were finally killed.

Partial Nephrectomy.

In 50 larvæ the glandular portion only of the right or left pronephros was extirpated. The larvæ averaged 6.5 mm. in length. At the end of 24 hours, only three larvæ showed indications of edema and, in all three, the edema was confined chiefly to the side from which the pronephros had been removed. All the larvæ showed swelling and hypertrophy of the remaining uninjured pronephros.

38 hours after the operation half the larvæ of the culture was edematous. In the early stages, the swelling was almost always on the side from which the kidney had been removed. Dissection of the pronephros remaining showed the tubular complex to be much hypertrophied and distended with fluid. A comparison of the pronephros of such larvæ with those from normal controls of the same age showed a difference in size, though we should expect it in such cases.

The onset of edema in the larvæ of this group was very much slower and less marked than in any other culture of the larvæ. In fact, fully one-half showed only slight edema, which was confined chiefly to a slight turgor of the submaxillary lymph sinus. 50 hours after the operation ten larvæ were counted which showed no edema whatever, and no indication of rupture of the body wall. It is clear that the mesonephros of these larvæ was beginning to function.

Removal of Portion of Wolffian Ducts.

A series of 62 larvæ was operated upon; the nature of the experiment varied from that just described in that, instead of extirpating the glandular portion of the pronephros, sections of its duct, on either side, were cut out just cephalad to the cloacal opening. Larvæ of 7 mm. in length were used in this experiment.

20 hours after the operation, examination of the larvæ showed great swelling of the glandular part of the pronephros. The swelling protruded as large knots of edematous tissue on either side of the head region. Edema appeared in most of the larvæ a few hours later. A curious condition now resulted. In addition to the marked turgor of the head region, and to the two swellings on either side of the body, representing the hypertrophied pronephros, there was a large edematous swelling on either side of the larva in the posterior part of the body at the place where the cut ends of the Wolffian ducts ended blindly in the tissues. Evidently the pronephros was functioning, but the fluid was being forced into the tissues of the posterior end of the body.

An operation of a somewhat different nature from those recorded above was also attempted. Twenty-five larvæ, measuring 7 mm. in length, were used. The Wolffian ducts on either side were severed, just cephalad to the cloacal opening, and dissected out anteriorly well up toward the glandular portion of the pronephros. The ducts were permitted to hang outside the larvæ through the incision made in the body wall. Considerable mortality resulted, no doubt due to the severity of the operation and consequent destruction of such important vascular organs as the posterior cardinals and dorsal aorta. Two-thirds of the larvæ died during the first 24 hours, and at the end of 36 hours only three remained alive. When examined, they showed practically no edema, except a slight turgor of the head lymph sinuses and swelling of the glandular portion of the kidney. The Wolffian ducts still hung outside the body and were apparently functioning as excretory ducts, despite their abnormal position. The incision in the body wall at the point where the ducts protruded had not healed at this time. Complete healing of the wound would no doubt have pinched off the ducts or else have closed them by pressure. All

the larvæ died, and it was impossible to keep them alive long enough to see whether the wound would have healed, leaving the ducts functional.

It was observed during the course of the experiments that the onset of the edema appears to be coincident with the opening of the Wolffian ducts into the cloaca. It is at about this time also that the yolk mass begins to undergo rapid resorption. Apparently water is not rapidly absorbed through the integument until the yolk mass undergoes considerable diminution.

In this connection, it was suggested by Professor McClure that the onset of edema in the nephrectomized larvæ should coincide with the initiation in the embryonic body of a continuous lymph flow through the lymphatics, between the periphery and the veins, since the attainment of this condition presents the first opportunity for water, absorbed through the skin and taken up by the lymphatics, to reach the kidneys by way of the lymphatic and blood vascular systems. This condition is established normally in frog and toad larvæ, according to McClure,[3] at about the time when the intestine has differentiated out of the yolk mass, and the yolk has undergone resorption. I feel sure that the onset of edema occurred at about this time in the nephrectomized frog larvæ.

The extirpation experiments recorded here show that any malfunctioning of the kidney, any block in its excretory canals, or complete loss of function, as for instance in nephrectomy, leads to an excessive accumulation of fluids in the lymph sinuses, tissues, and body cavity. They therefore confirm in a striking manner the results obtained by McClure and, as far as the Anura are concerned, it can now be regarded as an established fact that edema may arise from a block in kidney function.

I wish to acknowledge my indebtedness to Professor McClure for many helpful suggestions and for the interest he has shown in the work.

[3] McClure, C. F. W., On the behavior of *Bufo* and *Rana* toward colloidal dyes of the acid azo group, Memoirs of The Wistar Institute of Anatomy and Biology, No. 8, Philadelphia, 1918, 48; On the provisional arrangement of the embryonic lymphatic system, *Anat. Rec.*, 1915, ix, 281.

Addendum.—After this article was in press, the work of Miss Ruth B. Howland[4] came to my attention. The results obtained by Miss Howland on the embryos of *Amblystoma* are essentially similar to those described above by me for the frog. It is an agreeable duty for me to ackowledge, however, the full priority of Miss Howland's results.

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[4] Howland, R. B., On the effect of removal of the pronephros of the amphibian embryo, *Proc. Nat. Acad. Sc.*, 1916, ii, 231.

ANTAGONISM BETWEEN ALKALOIDS AND SALTS IN RELATION TO PERMEABILITY.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, March 23, 1919.)

Previous investigations[1] have led the writer to the view that salts which have opposite effects on permeability are able to antagonize each other. It seemed of interest to discover whether this is also true of antagonism between salts and alkaloids. Such antagonism has been reported by several authors, the most extensive investigation being that of Robertson.[2]

In order to compare the antagonism between alkaloids and salts with that between salts and salts, experiments were carried out under precisely the same conditions in both cases. These experiments were made by determining the electrical conductivity of *Laminaria* in a manner previously described.[3]

Taking a typical case of salt antagonism, that of NaCl and $CaCl_2$, we find that NaCl produces an increase of permeability while $CaCl_2$ produces a decrease (followed by an increase).[4] We may therefore expect that if alkaloids antagonize NaCl they will produce a decrease in permeability.

The alkaloids studied were nicotine, caffeine, and cevadine. They were added in varying amounts to NaCl 0.52 M,[5] and their effect upon the electrical conductivity of *Laminaria* was determined.[6]

[1] Osterhout, W. J. V., *Science*, 1915, xli, 255.

[2] For the literature see Robertson, T. B., *J. Biol. Chem.*, 1906, i, 507. *Ergebn. Physiol.*, 1910, x, 238, 311. Also Szücs, J., *Jahrb. wiss. Bot.*, 1912, lii, 85.

[3] Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 485.

[4] Osterhout, W. J. V., *Science*, 1912, xxxv, 112.

[5] All the solutions had the same conductivity as sea water.

[6] For the precautions used in preparing the material see Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533.

The results obtained with nicotine are shown in Fig. 1. The lower curve shows that after 18½ hours the resistance of the tissue has dropped to 10 per cent (the death point) in 0.52 M NaCl, as well as in 0.52 M NaCl to which sufficient nicotine has been added to make

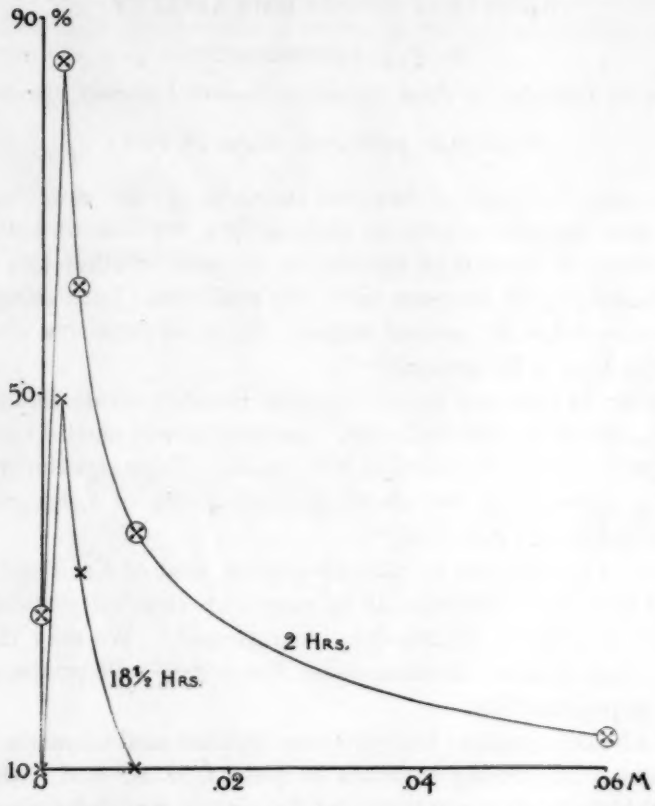

FIG. 1. Curves showing antagonism between NaCl and nicotine. Ordinates represent net electrical resistance of *Laminaria* (expressed as per cent of the normal); abscissæ represent concentrations of nicotine added to 0.52 M NaCl. The resistance of the control at 18½ hours was 94 per cent. Average of two experiments; probable error less than 3 per cent of the mean.

the concentration 0.01 M. In NaCl 0.52 M plus nicotine 0.002 M the resistance has dropped to 49.5 per cent (*i.e.*, the tissue is about half dead). It is evident that nicotine antagonizes the action of

NaCl by inhibiting the fall of resistance which occurs in pure NaCl. The upper curve (2 hours) shows even more pronounced antagonism.

The results with caffeine (Fig. 2) are similar except that the curve does not fall as rapidly with increasing concentrations of alkaloid.

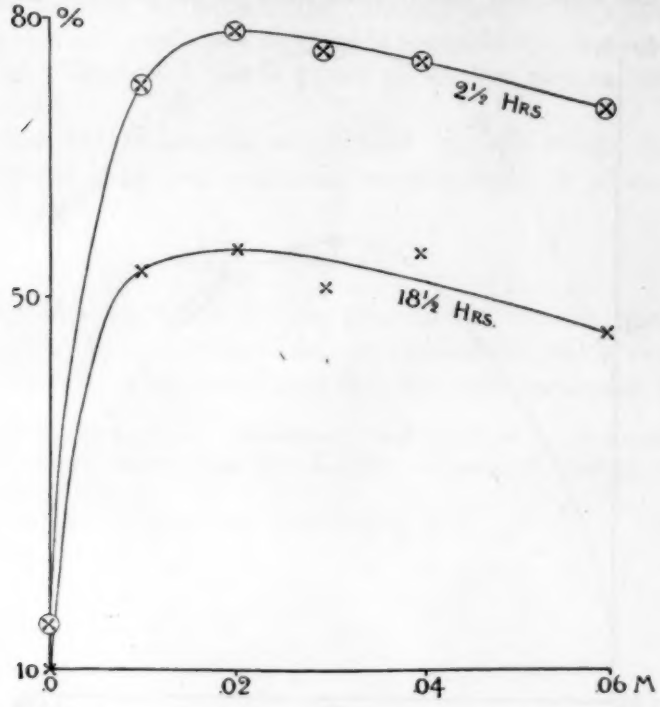

Fig. 2. Curves showing antagonism between NaCl and caffeine. Ordinates represent net electrical resistance of *Laminaria* (expressed as per cent of the normal); abscissæ represent concentrations of caffeine added to 0.52 M NaCl. The resistance of the control at 18¼ hours was 96 per cent. Average of two experiments; probable error less than 5 per cent of the mean.

With cevadine (Fig. 3) the curve falls much more rapidly, the maximum being in the neighborhood of 0.005 M cevadine sulfate. Here death is more rapid, the tissue being killed in 18 hours or less, even in the most favorable solution.

The experiments with cevadine were carried out during the day at 15 ± 2°, and the time curves in the various solutions follow more

or less closely a monomolecular course. In the case of nicotine and caffeine (where the experiment ran during the day and the following night) this is not the case, except in the earlier part of the reaction. This is perhaps explained by the fall of temperature which occurred during the night and retarded the speed of the process. It should

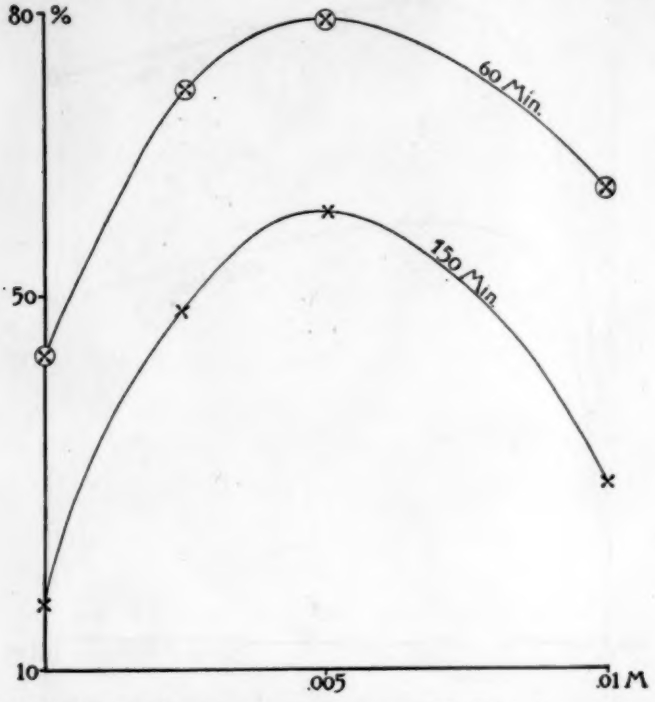

FIG. 3. Curves showing antagonism between NaCl and cevadine sulfate. Ordinates represent net electrical resistance of *Laminaria* (expressed as per cent of the normal); abscissæ represent concentrations of cevadine sulfate added to 0.52 M NaCl. The resistance of the control at 150 minutes was 100 per cent. Average of two experiments; probable error less than 5 per cent of the mean.

be noted that all the experiments in any set were begun at the same time, so that all shared equally in the variations of temperature; in consequence the form of the antagonism curve is not greatly affected by such variations.

In order to determine whether these alkaloids produce a decrease in permeability they were added to sea water. The experiment was

not successful in the case of nicotine, owing to the formation of a visible precipitate, which was apparently due to the presence of calcium and magnesium in the sea water. In the case of caffeine (0.01 to 0.04 M) and of cevadine sulfate[7] (0.0006 to 0.0025 M) a distinct decrease in permeability was found (as shown by the rise in resistance); this was followed by an increase. In this respect they resemble $CaCl_2$ which also produces a decrease in permeability when added to sea water. The same is true of typical anesthetics, such as ether and chloroform.[8]

The idea that substances which have opposite effects on permeability can antagonize each other seems to apply to alkaloids as well as to salts.

SUMMARY.

Certain alkaloids which decrease permeability are able to antagonize NaCl. In this respect they resemble salts which decrease permeability, for it has been shown that such salts antagonize NaCl.

[7] This is regarded as two molecules of cevadine united to one molecule of H_2SO_4. It was purchased from Merck under the name of veratrine sulfate $(C_{32}H_{49}NO_9)_2 . H_2SO_4$.

[8] Osterhout, W. J. V., *Bot. Gaz.*, 1916, lxi, 148.

A NEW APPARATUS FOR MEASURING SURFACE TENSION.

By P. LECOMTE DU NOÜY.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 31, 1919.)

Surface tension is probably one of the most difficult phenomena to measure. Although a great deal of ingenuity has been spent for almost a century in devising accurate techniques, the figures obtained deviate more from each other for the same substance, according to different authors, than any other constant characterizing the substance. It is well known that the two classes of methods of measurement, the static and the dynamic give entirely different results when applied to the same liquid. The following figures illustrate these differences.

	Dynamic.	Static.
Water	73	73
Sodium oleate	26	79
Heptylic acid	54	68

The static values were obtained by the capillary tube method, and the dynamic values by the oscillating jet method. Nevertheless, under given conditions, all the methods give practically concordant values in the case of pure liquids, but not in the case of solutions.

With the static methods (among which are weight of hanging drops, rise in a capillary tube, adhesion of a disc to the surface, excess of pressure in a spheric bubble produced in a liquid, direct measurement of curvature of the surface), the following figures are obtained for the surface tension of water.

	Dynes. cm.
Method of pulling off a ring	81.0
" " capillary tube (air)	75.5
" " " " (water vapor)	73.0

Some authors give 82 for the first method, others, 80, and for the third, some give 68.

Temperature plays an important part. It may be represented by the simple formula:

$$A = A_0 (1-\alpha t)$$

The cleanliness of the apparatus containing the liquids and of the measuring devices is extremely important, for a minute trace of greasy substance spoils the results.

The importance of the action of surface tension in biological phenomena is well known, but all the techniques of measurement are either complicated (static methods: capillary undulations, drop rebounding, capillary jets, rebounding jets), or long (drop method), and it is desirable to have a simple apparatus by means of which the surface tension, and especially the variation of surface tension of a given liquid, could be readily measured, with sufficient accuracy. For this reason the apparatus to be described has been designed (Fig. 1). There is no new principle in it; it is based upon adherence of a ring, or of any other design, to the liquid (Weinberg). It is simply a torsion balance, but instead of measuring the tension by means of weights (which is time-consuming, and makes two readings necessary), the torsion of the wire is used to counteract the tension of the liquid film and to break it. A single reading on a dial indicating the degree of torsion of the wire gives a figure, which, if the apparatus has been previously standardized with water, gives the surface tension of the liquid by a simple proportion. According to the fact that the torsion of the wire for water, which has the highest surface tension, is only 72°, we can assume that, within these limits, the strain of the wire is proportional to the angle of torsion, so that no table of correction is needed (see Fig. 2). Of course, the apparatus may be used for standard measurements also, by simply forcing the lever to come back to its former position by means of weights after the tearing of the membrane has taken place. By using the comparison method, the figures are reliable and constant, and the time necessary to make one measurement does not exceed 15 to 30 seconds. A very small quantity of the liquid is required,—about 1 cc. in a watch-glass,—but the apparatus could be fixed in such a way that it would need only 0.5 cc.

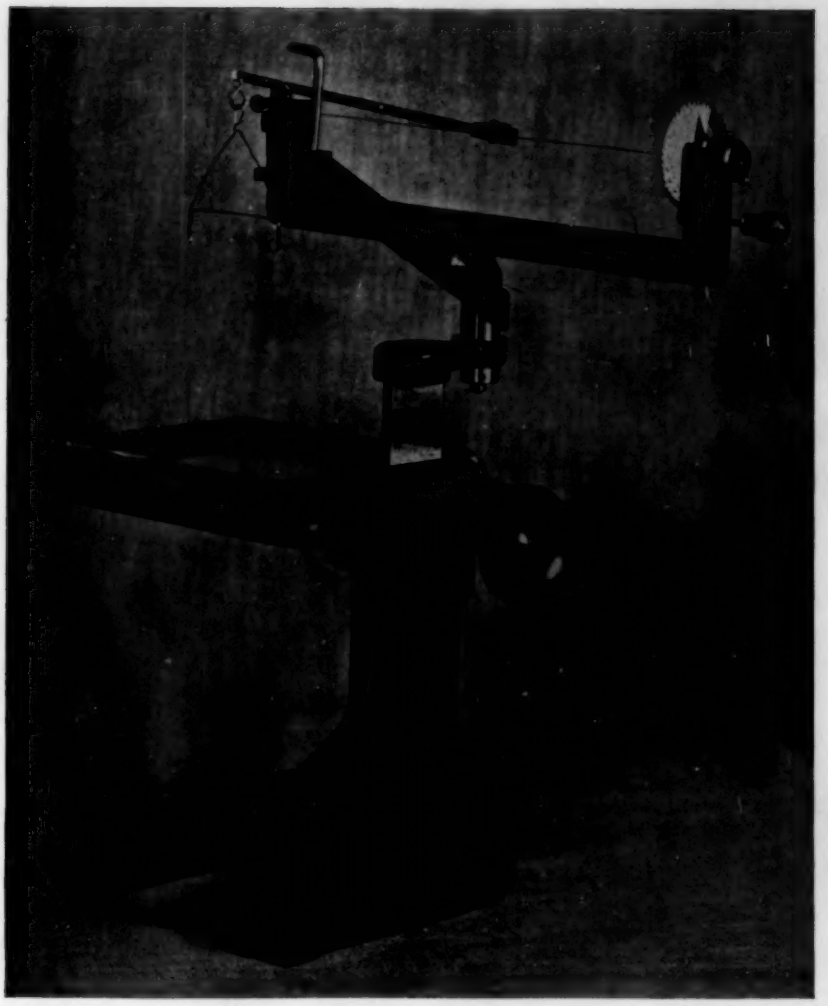

FIG. 1.

The tension of the wire can be adjusted. The torsion is controlled by a gear, which moves very slowly. The dial shown in Fig. 1 is attached to the wire and turns with it. A fixed needle indicates the angle of torsion. The apparatus described is home-made, and would be greatly improved by using a large dial and a worm-gear for controlling the torsion.

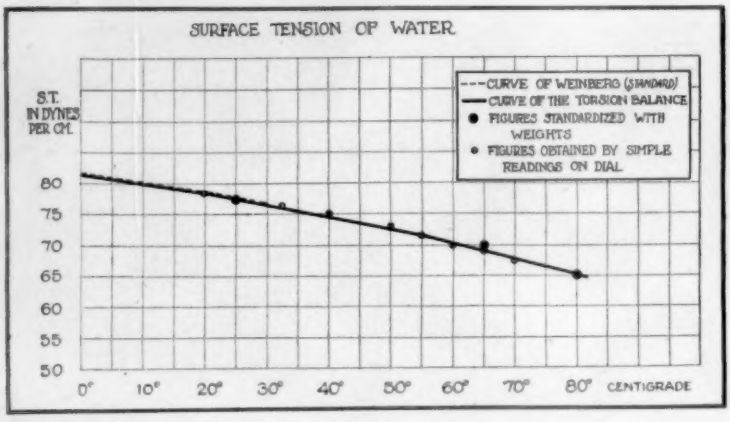

FIG. 2.

With the apparatus described above, with a platinum ring, the circumference of which is equal to 4 cm., 77 dynes per cm. were found for the surface tension of ordinary distilled water, at 25°C., 81 dynes at 0°, and 65 dynes at 80° (see Fig. 3).

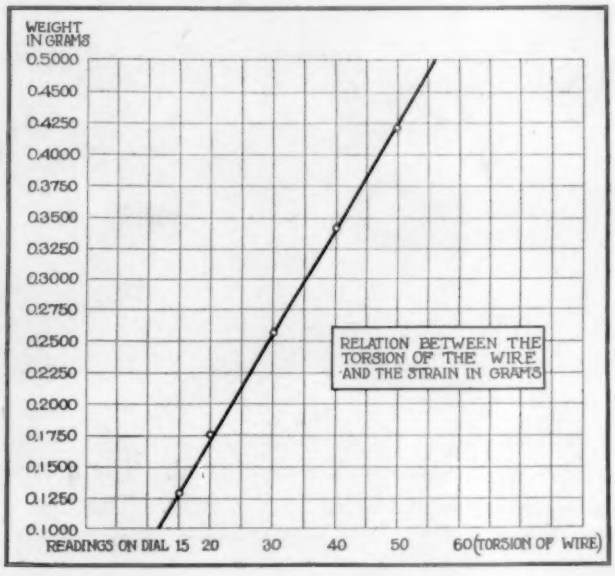

FIG. 3.

RELATION BETWEEN METAMORPHOSIS AND OTHER DEVELOPMENTAL PHENOMENA IN AMPHIBIANS.

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In 1833 von Schreibers[1] reported that in cold springs the larvæ of salamanders sometimes refrain from metamorphosis for a considerable length of time; upon dissecting such larvæ he found the remarkable fact that the sex organs had reached maturity before metamorphosis had taken place. Since that time, similar observations have been made repeatedly; but it was due especially to De Filippi's[2] discoveries, communicated in 1861, that the attention of biologists was directed to this phenomenon. De Filippi found a pond in the Formazza valley, near Andermatten, which was inhabited by numerous individuals of the species *Triton alpestris*. The majority of them had metamorphosed; but about forty were still in a larval condition and possessed gills. They were conspicuous not only by their unusually large size as compared with normal larvæ of this species, but also by the presence of mature sex organs. Soon after De Filippi's communication, Dumeril[3] reported his remarkable experiments on amphibians—probably the Mexican axolotl—in which it was demonstrated that the specimens in question were larvæ of the species *Ambystoma tigrinum* and not adult animals, and yet the larvæ not only possessed mature sex organs, but actually propagated in the laboratory while still in the larval condition.

These observations proved conclusively that the development of the sex organs in amphibians is independent of metamorphosis. Expressed in terms of Gudernatsch's[4] discoveries, according to which metamorphosis is caused by the action of definite substances, this would mean that the substances bringing about development of the sex organs are not identical with the substances causing metamorphosis. Recently the same condition has been found to prevail among

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[1] von Schreibers, *Oken's Isis*, 1833, 527.

[2] De Filippi, F., *Arch. per Zool. l'Anat. e Fisiol.*, Geneva, 1861, i, 219 (quoted by Wolterstorff).

[3] Dumeril, A., *Nouvelles Arch. Mus. Histoire Nat. Paris*, 1866, ii, 265; *Ann. sc. nat. Zool.*, 1867, vii, 229.

[4] Gudernatsch, J. F., *Arch. Entwcklngsmechn. Organ.*, 1912–13, xxxv, 457.

the tailless amphibians. Allen[5] has shown that extirpation of the thyroid in the larvæ of *Rana pipiens*, though it prevents metamorphosis, does not retard development of the sex organs, and Swingle's[6] experiments prove that feeding thyroid to tadpoles, though it enforces metamorphosis, does not accelerate development of the germ cells.

The fact that the germ cells in amphibians are independent of metamorphosis as regards their development has recently given rise on the part of Professor Allen[5] to renewed consideration of Weismann's hypothesis postulating the existence of a fundamental difference between the germ plasma and the somatic plasma, since the observations made in amphibia may be interpreted to mean that the germ plasma is independent of the somatic plasma. This interpretation, however, would be correct only if it could be shown that the independent behavior during development is peculiar only to the germ cells, and that consequently the soma as a unit is opposed to the germ cells as an independent unit. But we will show, in the present paper, that upon further analysis of amphibian development, it is found that the so called soma, in the Weismannian sense, does not exist; for it is possible to demonstrate that in salamanders this soma can be made to disintegrate into a number of organs which, like the sex organs, are independent of metamorphosis and, moreover, are independent of each other as regards their development. Furthermore, the nature of metamorphosis will have to be defined more accurately to mean not the development of the animal as a whole, but the development of certain organs of the amphibian organism. The remarkable feature in the development of amphibians is not the independence of the germ plasma from the somatic plasma, but the independence of various groups of organs from one another, due to the fact that the development of each of these groups is controlled by substances different from those controlling the other groups, and that each of these substances separately may be supplied to or withheld from the organism by the experimenter at will, either directly or by means of changing the environmental conditions.

[5] Allen, B. M., *J. Exp. Zool.*, 1917–18, xxiv, 499.
[6] Swingle, W. W., *J. Exp. Zool.*, 1917–18, xxiv, 521.

Relation between the Reduction of the Gills, the First Skin Shedding, and Metamorphosis.

It has become customary to apply the term metamorphosis to the processes which can be enforced by thyroid treatment. Though it is claimed frequently that in tadpoles the action of iodine can be defined accurately, it seems that certain phenomena have been included as a result of iodine action which in reality are not due directly to this action. It will be shown that the effect of iodine can be determined most accurately in salamanders, at least as regards two organs, the gills and the skin, while the development of several organs can be shown to be independent from the iodine mechanism.

In salamanders, two phenomena are most conspicuous during metamorphosis; the first shedding of the skin and the reduction of the gills.

In temperatures of from 20–30°C., the first shedding of the skin never takes more than 24 hours and frequently only from 2 to 5 hours. In lower temperatures it may take from 1 to several days. But in both cases the beginning and the end can be determined with accuracy if the animals are watched carefully. It is thus possible to observe accurately the time relation which exists between the first shedding of the skin and other developmental phenomena. While several of the latter phenomena have been observed to take place either before or after the first shedding of the skin, as will be discussed below, the time relation between the shedding of the skin and the reduction of the gills is constant and unchangeable. The condition of the gills (external in salamanders) varies greatly and is influenced much by the quantity of food available to the larvæ, as Powers[7] has stated. Well nourished larvæ possess large gills with long fringes; in poorly nourished animals the stems as well as the fringes become short. But in whatever state the larvæ may be kept, the gills are always characterized by the possession of stems as well as fringes. In metamorphosis, however, the gills are reduced to short stubs without fringes. The transition from the state with fringes into that without fringes is sudden and definite, and requires only a few hours.

[7] Powers, J. H., *Am. Nat.*, 1903, xxxvii, 385.

Many hundreds of salamander larvæ belonging to the species *Ambystoma maculatum*, *Ambystoma opacum*, *Ambystoma tigrinum*, and *Eurycea rubra* have been observed under the most varying conditions during metamorphosis; but in not one instance were the gills reduced to mere stubs without fringes before the shedding of the skin had taken place. This time relation is observable even under conditions of the greatest acceleration. Since it will become evident, and has already been pointed out above that a similarly constant succession in time between the two phenomena in question and other developmental phenomena does not exist, we are forced to assume that a relation exists between the shedding of the skin and the reduction of the gills, which does not exist between these phenomena and other developmental processes. Evidently both these phenomena must have a common cause, and, indeed conditions which retard or prevent the shedding of the skin also retard or prevent the reduction of the gills, while conditions which accelerate or enforce the shedding of the skin also accelerate or enforce the reduction of the gills. Moreover, it can be shown that both phenomena are enforced by the application of iodine and inhibited by the lack of iodine; and consequently they must be considered as constituting part of the amphibian metamorphosis. Numerous experiments have been carried out to test the action of iodine upon the shedding of the skin and the reduction of the gills; two are described below.

A series of six larvæ of *Ambystoma opacum* (W_I 1917) were kept in ordinary tap water at approximately 25°C. and fed on earthworms. 5 weeks after hatching, the larvæ had attained a length of 29.6 mm. and were in an early larval stage. At this time they were placed in a 0.02 per cent solution of Bayer's iodothyrin in tap water. 8 or 9 days later, the skin was shed and the gills were reduced rapidly to stubs without fringes, while the control larvæ needed several weeks more to undergo the same changes. This effect of iodine upon skin shedding and the reduction of the gills was particularly conspicuous as the development of other organs, *e.g.* the legs, did not make any progress. Thus the phenomena of skin shedding and reduction of the gills surely are caused by the action of iodine, and constitute what we must call metamorphosis.

In a previous paper[8] it has been demonstrated that the feeding of the thymus gland to larvæ of *Ambystoma opacum*, *Ambystoma maculatum*, and *Ambystoma tigrinum* frequently retards and in rare cases even inhibits metamorphosis, and that this inhibitory action of the thymus diet is due to the fact that iodine is either completely absent from the thymus or present in amounts so small as to make it impossible for metamorphosis to take place at a normal time. In the thymus-fed larvæ not only the reduction of the gills but also the shedding of the skin is either retarded or completely inhibited, and yet other organs may develop normally and reach an almost adult condition. This proves that while the development of the latter organs is independent of the iodine action, both the skin shedding and the reduction of the gills cannot take place in the absence of iodine.

Relation between Development of Skin Coloration and Metamorphosis.

Among the organs whose development is independent of metamorphosis, the structures which give rise to the coloration of the skin are most conspicuous. Since the coloration of the skin is easily observable without killing the animal, the development of this character can be used with great advantage to demonstrate its relation to metamorphosis. The color development of the skin is most variable as regards its time relation to metamorphosis. This relation is subject to change under a number of different conditions; the development of the skin coloration is not produced by the substances causing metamorphosis, for it takes place without the action of iodine.

Though careful studies on the subject in question have not been carried out thus far, the remarkable fact that gill-bearing larvæ may exhibit the coloration of metamorphosed animals has been observed repeatedly. In 1891 von Bedriaga[9] described in detail the coloration of a number of larvæ of tailed batrachians and frequently emphasized in this paper that neotenous larvæ of the genus *Triton* may possess colors similar to those of metamorphosed animals. Similarly

[8] Uhlenhuth, E., *J. Gen. Physiol.*, 1918–19, i, 305, 473.

[9] von Bedriaga, J., *Zool. Anz.*, 1891, xiv, 295, 301, 317, 333, 349, 373, 397.

Wolterstorff[10] observed that the coloration continued to develop in urodelan larvæ whose metamorphosis had been checked; he mentioned that in this phenomenon we seem to deal with a noteworthy case of organic correlation. He probably thought, however,—erroneously as will be shown later on—that the development of the skin pigmentation is conditioned by the development of the sex organs, a correlation existing between these two organs. Later on Kammerer[11] observed a larva of *Salamandra maculosa* which had developed the skin pattern of the adult a year and a half before metamorphosis took place. Similar phenomena have been observed also among the larvæ of *Salientia*. Dickerson,[12] for instance, in a description of the metamorphosis of *Rana clamitans*, says: "The variation in the size, color, and markings of the changing tadpoles is very great. Most curious is the fact that some tadpoles show the lateral folds and the coloring of the adult male or female long before the change is completed, while others take on the frog form entire before the lateral folds are well developed or before the sexual coloring is evident."

The following experiments show that the development of the skin coloration cannot be prevented by the absence of iodine from the food, nor can it be enforced by the direct application of iodine; consequently it is independent from metamorphosis and may take place either before or after metamorphosis.

Four series of larvæ of *Ambystoma opacum*, all hatched from eggs of one female, were kept at approximately 25°C.; two series (*A* and *E*, 1916) were fed earthworms, the other two (*B* and *F*, 1916) were fed calf's thymus. In Series *A* and *E* the gray network of the adult commenced to develop as soon as the animals had metamorphosed (Figs.

FIGS. 1 to 4. Development of the adult coloration of the skin in worm-fed and thymus-fed animals of *Ambystoma opacum*. In worm-fed animals the gray network develops after metamorphosis (Figs. 1 to 3), in thymus-fed animals, the metamorphosis of which is retarded, it develops before metamorphosis (Fig. 4). Photographed from water color paintings made from the living specimens. Natural size.

[10] Wolterstorff, W., *Zool. Garten*, 1896, xxxvii, 327.

[11] Kammerer, P., *Arch. Entwcklngsmechn. Organ.*, 1904, xvii, 165.

[12] Dickerson, M. C., The frog book, New York, 1907, 205.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 1. *Opacum* 1916, *A* 4. Worm-fed metamorphosed animal, 2 days after metamorphosis; gray network visible on the snout; rows of larval spots still present.

FIG. 2. *Opacum* 1916, *A* 3. Worm-fed metamorphosed animal, 2 weeks and 3 days after metamorphosis. Gray network fully developed, but rows of larval spots still present on back.

FIG. 3. *Opacum* 1916, *A* 3. Worm-fed metamorphosed animal, 6 weeks after metamorphosis. Gray network fully developed, rows of larval spots have disappeared.

FIG. 4. *Opacum* 1916, *F* 3. Thymus-fed larva. Gray network fully developed; rows of larval spots have disappeared.

1 and 2), and was fully developed from 5 to 6 weeks after metamorphosis (Fig. 3). In Series *B* and *F* the gray network of the adult had started to develop before metamorphosis. In five larvæ whose metamorphosis was either checked completely or retarded to a considerable extent, the gray network reached a condition very similar to the adult condition as shown in Fig. 4.

These experiments have been repeated several times and have always furnished the same result; namely, that the development of the adult coloration can proceed in the absence of iodine, and can take place before metamorphosis has been accomplished. Moreover, in the above mentioned larvæ the sex organs were not in a mature condition; the development of the skin color is independent not only of metamorphosis but also of the development of the sex organs.

On the other hand, iodine treatment though it leads rapidly to metamorphosis does not advance the development of the skin coloration. This was shown most strikingly in the Series *Opacum* 1917, W_I. In these larvæ the iodothyrin treatment enforced metamorphosis from 8 to 9 days after the beginning of the experiment; the small and little developed larvæ shed the skin and reduced the gills rapidly. The coloration of the skin, however, which at this time was that of an early larval stage, did not change at all under the influence of the iodine; consequently metamorphosed animals resulted with the color of young larvæ.

Similar experiments were conducted with the species *Ambystoma maculatum*. If the larvæ were fed on earthworms and kept at 25°C., the isolated yellow spots developed from the yellow network after metamorphosis; if they were fed on thymus and kept at 25°C., metamorphosis was retarded, but the development of the yellow spots proceeded notwithstanding.

Evidently the development of the structures leading to the coloration of the skin are caused by substances which are not identical with the substances causing metamorphosis, nor are they identical with the substances causing development of the sex organs. So far then, we have found three groups of organs, each of which is controlled by a different substance, or rather a different set of substances, as will be discussed below.

Relation between Development of Legs and Metamorphosis.

The statement is usually made that in tadpoles iodine enforces development of the fore limbs and that consequently the limb development of amphibians is part of the amphibian metamorphosis. We have observed that in salamanders the development of the limbs, the fore limbs as well as the hind limbs, is completely independent of metamorphosis; it is not in any way caused or even influenced by the iodine mechanism. On the one hand, the legs of thymus-fed animals develop at the same rate and to the same degree as in worm-fed control larvæ. On the other hand, larvæ subjected to iodine treatment do not show any precocious development of their limbs. In Series *Opacum* 1917, W_I (described above), the hind limbs had developed only four toes when the larvæ were exposed to the iodothyrin solution. Though the iodothyrin, as mentioned above, caused rapid shedding of the skin and reduction of the gills to stubs without fringes, it did not bring about the development of the fifth toe of the hind limbs; thus metamorphosed salamanders resulted with only four toes on the hind limbs. Hence development of the legs is not caused by iodine, but by substances different from those which control metamorphosis.

In accordance with this, the salamander larvæ develop their legs normally if they are deprived of their thyroids even as early as the stage just before the beginning of the blood circulation; for Hoskins and Morris[13] mention that larvæ of *Ambystoma punctatum* after thyroid removal behave like the controls, except that they do not grow so well; but they do not mention that thyroidectomy prevented the legs from developing.

From this experiment it is quite evident that in contradistinction to what has been observed in frog tadpoles, in the larvæ of the caudate amphibians, leg development and metamorphosis are subject to two different factors, and that, consequently, leg development can occur even if no metamorphosis takes place, or may not occur even if metamorphosis takes place.

[13] Hoskins, E. R., and Morris, M., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv. 74.

It is remarkable, however, that in two so closely related groups of animals as the caudate and tailless batrachians, the same process should be caused by so different a mechanism. It is difficult to refute the suspicion that in the *Salientia* the development of the limbs may be initiated only indirectly by the iodine action, perhaps on account of some incidental anatomical structure which first must be broken down by the autolytic action of the iodine. And, in fact, there exists such a structure in the *Salientia*, which may be in the way of the development of the fore limbs at least. It is well known that in the frogs and toads the fore legs begin to develop long before they become visible externally; they develop enclosed in the gill chambers and covered by the skin of the body. Would it not be possible that the autolytic changes of the skin which finally lead to the first shedding of the skin and which are actually effected by the iodine action, must first be initiated by the iodine in order that the legs can be freed and full development can take place in the tadpoles? In this connection the experiments which Lenhart[14] performed on tadpoles in order to test the action of thyroid glands of varying iodine content may be mentioned. He noticed that with increasing iodine content the absorption of the tail takes place more and more quickly while differentiation, on the contrary, becomes less, taking the development of the limbs as expression of the differentiation. This result can be understood only if we assume that the autolytic processes alone leading to absorption of the tail, shedding of the skin, and metamorphosis, are caused directly by the action of the iodine. If the time between the beginning of the autolytic processes and their final result, the shedding of the skin, is long enough, the legs have time to develop after they have been freed, before metamorphosis takes place. But if by means of strong iodine concentration this interval becomes much shortened, the legs have no time to differentiate before metamorphosis occurs, the iodine, the concentration of which was increased artificially, acting more strongly than the substances causing leg development, which were not increased artificially.

That leg development is not a part of the amphibian metamorphosis is also demonstrated by the fact that in certain amphibians,

[14] Lenhart, C. H., *J. Exp. Med.*, 1915, xxii, 739.

such as the cæcilians, legs are completely absent in the adult form and yet a normal metamorphosis takes place in these amphibians.

There are still other organs the development of which does not seem to be caused by the iodine action in metamorphosis. It has been observed by Cope[15] and confirmed later by Powers[16] that in *Ambystoma tigrinum* under certain conditions metamorphosis may take place without the tongue and the larval arch of the palatal teeth developing the adult condition. From this may result metamorphosed animals without tongue and with the larval arch of the palatal teeth persisting. The substances causing metamorphosis apparently do not influence the development of the two organs in question. Thus it seems that at least six chemical mechanisms are present in the amphibian organism, one for metamorphosis, one for the skin coloration of the adult, one for the sex organs, one for the legs, one for the tongue, and one for the palatal teeth. The remarkable feature in the amphibian development is that each of these mechanisms can act independently without influencing the others; the organism as a whole, as an organic individuality, seems to have no control over the harmonic development of these groups of organs, since the chemical mechanisms separately respond to conditions which are not located within but without the organism. We will see now that one of these conditions is the temperature, which affects the rate of some of these mechanisms in a different way and consequently brings about unequal development of various parts of the body.

Influence of Temperature upon Metamorphosis and Coloration of Ambystoma tigrinum.

If the coloration of the skin is caused by substances different from those which cause the amphibian metamorphosis, we can understand the influence of temperature upon the coloration of *Ambystoma tigrinum* as observed in the following experiments.

Two sets of larvæ of *Ambystoma tigrinum*, *Tigrinum* 1917, Series *S* and *U*, were kept at approximately 25° and 15°C. respectively; other-

[15] Cope, E. D., The batrachia of North America, Washington, 1889, 73. (*Bull. U. S. Nat. Mus., No. 34*).

[16] Powers, J. H., *Univ. Studies Univ. Nebraska*, 1907, vii, 197.

wise they were kept under exactly like conditions. The eggs from which they hatched in the laboratory were collected in Syosset, Long Island, in the spring of 1917; they were all taken from one single egg mass and were consequently laid by the same female. Soon after hatching the larvæ of both sets were fed on worms, which were used as food throughout the whole experiment. Each set consisted of six specimens; five of these metamorphosed in Series *S* and four in Series *U*. In Series *S*, metamorphosis took place 11 weeks and 6 days after hatching, in *U*, 22 weeks and 2 days after hatching.

In both series the color of the larvæ before metamorphosis was an even greenish black.

In Series *S* numerous yellowish spots of small size became visible all over the back as soon as the larvæ had shed the skin (Fig. 5). In Series *U* the larval color was retained after metamorphosis and no spots appeared; metamorphosed animals with a larval coloration resulted (Fig. 6).

In Series *S* the yellow spots soon began to increase considerably in size; in Series *U* no yellowish spots appeared before the 2nd week after metamorphosis, when a small number of tiny sepia colored specks made their appearance.

In Series *S* the yellowish spots had increased so much in size several weeks after metamorphosis that they became confluent here and there forming yellow blotches; no further changes took place in Series *U*. Fig. 7 shows an animal of Series *S* 13 weeks and 2 days after metamorphosis; the spots have fused to form blotches and bands of yellowish color. Fig. 8 shows an animal of Series *U*, 22 weeks and 3 days after metamorphosis; the spots are still small and few in number.

The final coloration of the adults is shown in Figs. 9 and 10. In Fig. 9 an animal of Series *S* is shown 33 weeks and 2 days after metamorphosis; very little of the black background is left, most of it being filled in by the large yellowish bands; in the animals kept at low temperature just the opposite occurs, as is shown in Fig. 10, 50 weeks and 5 days after metamorphosis. The number and size of the yellowish spots have increased only slightly.

These experiments show that the occurrence of banded and spotted individuals in *Ambystoma tigrinum* is not due to the existence of two different races, since in our experiments all animals were derived

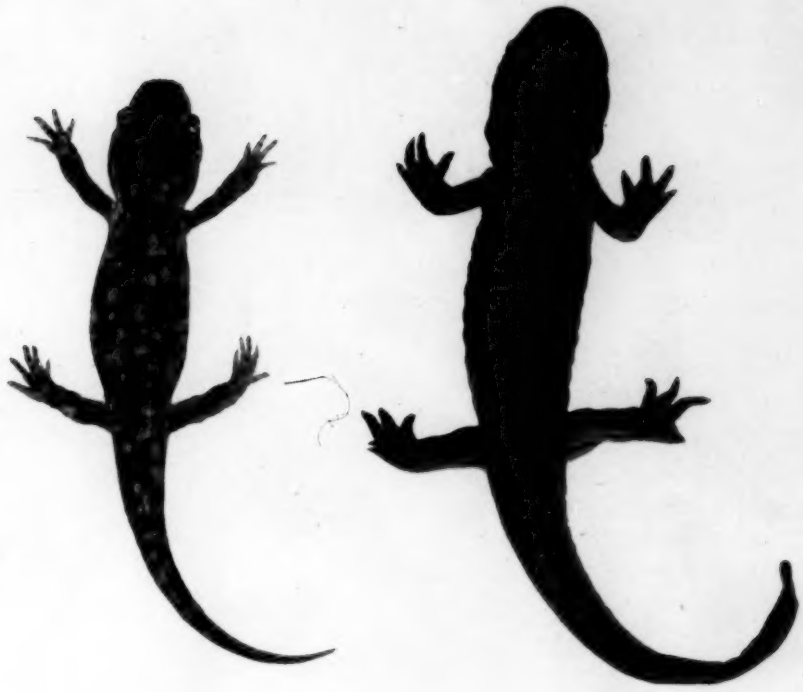

FIG. 5. FIG. 6.

FIGS. 5 to 10. In *Ambystoma tigrinum* the development of the yellowish spots is more retarded at low temperature than metamorphosis, and the development of the yellowish bands is completely inhibited. Fig. 6 photographed from live specimen. Fig. 10 photographed from water color painting made from preserved specimen. Rest of figures photographed from water color paintings made from live specimens. Natural size.

FIG. 5. *Tigrinum* 1917, *S* 3. 25°C., 12 days after metamorphosis. Numerous small yellowish spots.

FIG. 6. *Tigrinum* 1917, *U* 4. 15°C., 11 days after metamorphosis, larval coloration. No spots.

from one female. Furthermore, the difference in coloration as observed here is not due to the influence of light but to the influence of temperature. The action of the temperature is manifested in two ways: first, in low temperature the development of the yellow spots

FIG. 7.

FIG. 8.

FIG. 7. *Tigrinum* 1917, *S* 4. 25°C., 13 weeks and 2 days after metamorphosis. Yellowish spots fused to yellow blotches and bands.

FIG. 8. *Tigrinum*, 1917, *U* 4. 15°C., 22 weeks and 3 days after metamorphosis. Few small yellowish spots; no bands.

Fig. 9.

Fig. 10.

Fig. 9. *Tigrinum* 1917, *S* 4. 25°C., 32 weeks and 2 days after metamorphosis. Only little is left of the dark background, most of the space being taken up by the yellowish bands.

Fig. 10. *Tigrinum* 1917, *U* 4. 15°C., 50 weeks and 5 days after metamorphosis. Only small yellowish spots present; no bands have developed.

is more retarded than metamorphosis; second, the development of yellow bands is inhibited permanently in low temperature.

The first phenomenon can be explained as follows: In a previous paper it was shown that one of the substances causing metamorphosis, the excretor substance, is evolved during chemical processes, the rate of which depends on the rate of the processes leading to growth. Since the temperature coefficient of the excretor-forming processes is greater than that of the growth processes, the formation of excretor substance is decreased more than the rate of growth if the larvæ are kept at low temperature, and consequently the relation between size and metamorphosis is changed, the larvæ being larger at the time of metamorphosis when kept at low temperature than when kept at high temperature. Since the temperature coefficients of different physiological processes are frequently different, we might expect that the temperature coefficients of the processes causing metamorphosis are different from those of the processes causing development of the structures which give rise to the skin coloration of the metamorphosed animal. If this were so, we should expect that the time relation between metamorphosis and the development of the skin color should be changed not only by feeding or omitting substances which cause precocious metamorphosis but also by keeping the animals at low temperatures.

This is actually the case with the development of the yellowish spots in *Ambystoma tigrinum*. The retardation of the appearance of the yellowish spots as compared with metamorphosis can be explained if we assume that the chemical processes causing the development of the spots have a higher temperature coefficient than the chemical processes causing metamorphosis.

In order to understand the second phenomenon, the inhibition of the development of the yellowish bands, we shall again refer to metamorphosis.

One of the cases of metamorphosis most difficult to explain is the occurrence of a perennibranchiate form in the species *Ambystoma tigrinum* in certain localities. On the basis of the results so far obtained in our experiments the perennibranchiate form of the *Ambystoma tigrinum* can be explained tentatively as follows.

Two reasons may be assumed to account for the inhibition of metamorphosis in *Ambystoma tigrinum*. First, it is possible that with progressing age certain changes occur in the organism which abolish the susceptibility of the larvæ to the action of iodine; but experiments have shown that even very old larvæ can be forced to metamorphose by direct application of iodothyrin.

Second, it is possible that with progressing age certain changes occur in the thyroid gland which make this gland progressively less susceptible to the action of the excretor substance. By this assumption the problem can be solved. The localities where the perennibranchiate form of *Ambystoma tigrinum* occurs are all located high in the mountains and consequently the temperature of the water in these lakes is very low. As in other cases it is possible that the temperature coefficient of the thyroid change is lower than that of the excretor formation, and that for this reason lowering of the temperature decreases the rate of the excretor formation more than it decreases the rate of the thyroid change. Thus at the time when the quantity of excretor required under normal conditions to induce thyroid excretion would be evolved, the thyroid would be in a state where this quantity would not any longer be sufficient to affect excretion. If the rate of the thyroid change which makes this organ less and less sensitive to the action of the excretor, and the rate of the excretor formation would be constant under these conditions, the difference between the quantities of the two factors would increase progressively, and metamorphosis would become more and more impossible.

The development of the yellow bands of *Ambystoma tigrinum* in low temperature is indeed very similar to the metamorphosis of the same species, both becoming inhibited permanently by low temperature; the only difference is that inhibition of the development of the yellow bands can be effected at a temperature somewhat higher than that at which metamorphosis can be inhibited. From this similarity we may conclude that the development of yellow bands in *Ambystoma tigrinum* is due to an interaction of factors similar to but not identical with those involved in metamorphosis. Possibly one of these factors is a structure which loses in susceptibility towards a

certain substance with progressing age, the temperature coefficient of that change being lower than that of the processes which produce the substance in question.

It is possible that mechanisms similar to the mechanism involved in the development of the skin color are responsible also for the development of the tongue and palatal teeth, both phenomena being checked definitely by low temperature, as pointed out by Cope[15] and Powers.[16]

DISCUSSION.

In previous experiments[17] it has been shown, by grafting organs from one larva to a larva of different age, that the development of certain stages of the eye and of the skin pigmentation is caused, like the processes of metamorphosis, by substances which are not produced in the organs themselves but reach them through the blood circulation. In this paper further proof of the existence of such substances has been found in experiments made by a different method. But at the same time it has been shown here that the substances which are causing development of these organs are not identical with the agents causing metamorphosis and consequently the development of these organs cannot be included under metamorphosis. It is certain that in the salamanders examined at least five groups of organs, the structures leading to pigmentation of the skin, sex organs, legs, tongue, and palatal teeth, are independent of each other and of metamorphosis. It is probable that at least six different chemical mechanisms exist in the amphibian organism each of them for another group of organs. Consequently it should be possible to change the succession in time of these developmental phenomena by feeding directly substances which contain or lack the agent for only one of them; this can be actually accomplished in the case of metamorphosis and development of the skin color by iodine treatment or thymus feeding. But the same result should be accomplished by keeping the larvæ in different temperatures, since the temperature coefficients are different for the chemical reactions constituting each of these mechanisms. This has

[17] Uhlenhuth, E., *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvi, 211; *Arch. vergl. Ophthalm.*, 1913, iii, 343; *Proc. Soc. Exp. Biol. and Med.*, 1917, xiv, 88; *J. Exp. Zool.*, 1917–18, xxiv, 237.

been shown to be actually the case for metamorphosis and coloration in *Ambystoma tigrinum*, and probably the same cause is responsible for the production of metamorphosed animals without tongue and with a larval arch of palatal teeth.

It is probable that two factors are causing metamorphosis, iodine and excretor substance. A third factor is probably involved in inhibition of metamorphosis at low temperature; namely, a change of the thyroid gland, making this organ progressively less susceptible to the excretor action, with progressing age. The inhibition of the development of the yellow bands at low temperature in the species *Ambystoma tigrinum* is so similar to the inhibition of metamorphosis by low temperature in the same species as to suggest that the mechanism causing this coloration is composed also of a number of factors interrelated in a way similar to that of the factors for metamorphosis. It would be interesting to find out whether one of these factors, as in metamorphosis, is an internal secretion.

The experiments reported here show that the independent development of the germ cells cannot be used to postulate an independent position of the germ plasma as compared with the somatic plasma, since other groups of organs which so far as known are not composed of germ plasma behave like the germ cells. The fact that in salamanders different groups of organs are evolved by the action of different substances seems to agree well with similar phenomena in plants, since it has been shown that the different organs of the plant are caused to develop separately and apparently without the control of what may be called an organic individuality, by the action of different substances, special substances, for instance, serving for development of leaves and roots.[18]

SUMMARY.

1. The difference in time existing between the first shedding of the skin and the reduction of the gills to mere stubs without fringes is constant and unchangeable, which indicates that the fundamental cause for both is a common one.

2. This common cause is the action of iodine, and consequently both phenomena constitute, or at least are part of, the metamorphosis of the salamanders.

[18] Loeb, J., *Bot. Gaz.*, 1915, lx, 249; 1916, lxii, 293; 1917, lxiii, 25.

3. The development of the adult skin coloration and of the legs may take place either before or after metamorphosis. Iodine cannot enforce either of these phenomena.

4. The same is true of the development of the sex organs.

5. Development of the tongue and palatal teeth can be checked even in animals in which metamorphosis takes place.

6. Consequently development of the skin coloration, as well as development of the legs, sex organs, tongue, and palatal teeth are all caused by substances not identical with the substances causing metamorphosis and, since they are also all independent of each other in their development, it is probable that special chemical mechanisms exist for the development of each one of these six groups of organs.

7. This assumption is also supported by the fact that the order of development in several of these organ pairs can be changed by a difference in temperature, which would indicate that the development of each of these groups of organs is caused by chemical reactions with different temperature coefficients.

8. That the germ cells can develop in amphibians either before or after metamorphosis does not mean that the germ plasma is opposed as a unit to the somatic plasma, since other organs which are believed to be part of the somatic plasma behave in this respect like the germ cells.

9. The noteworthy feature of the amphibian metamorphosis is that instead of being controlled and kept in harmony by the organic individual the development of at least six groups of organs is controlled separately by the action of probably six different chemical mechanisms, each of which can be stopped or enforced independently either by directly supplying the substances required or by causing an increased formation within the body by suitable temperatures.

SENSORY EQUILIBRIUM AND DARK ADAPTATION IN MYA ARENARIA.

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INTRODUCTION.

Animals which are sensitive to a sudden increase in illumination possess two outstanding characteristics. One is the ability of the organism to come into sensory equilibrium on continued exposure to a given intensity of light. The other is the restoration of its sensibility to this intensity by subjection to a lower intensity for a definite interval. The latter represents a process of dark adaptation; the former, by analogy, a process of light adaptation. The terms are not happy ones. However, since the "light-adapted" eye, and the "dark-adapted" eye are commonplaces of sensory physiology, they are retained.

The mollusk, *Mya arenaria* Linné, presents these two phases of its sensitivity in measurable form. It has therefore been possible to investigate them carefully and to suggest a photochemical system which underlies them. In a measure, this has already been sketched in for *Ciona intestinalis* (Hecht, 1918, *a*, *b*). The present investigation of *Mya*, however, serves as an analysis of the photochemical system in such quantitative detail as to increase materially its scientific value and its probable reality.

The purpose of this paper is threefold: first, to describe the general properties of the photic sensitivity of *Mya;* second, to analyze the data relative to dark adaptation and sensory equilibrium; and third, to record some experiments designed to test the validity of the photochemical system which is suggested as an explanation of these phenomena. The experimental work was done at Woods Hole, Mass., during July and August, 1918.

Sensitivity of Mya.

1. *Mya* retracts its double siphon when it is suddenly illuminated. The reaction time of any individual to a given intensity is constant. In addition, animals of the same size give about the same reaction time under similar conditions (see Table I). The figures in Table I represent three measurements of the reaction time of each of six animals to one intensity.

Table I shows that it is a safe procedure to consider all the values together in order to express the mean reaction time of the group of animals. Because of this it was possible to perform a single experiment on several individuals, rather than to repeat the same de-

TABLE I.

Light Intensity, 85 Meter Candles. Animals in Dark Room for 24 Hours.

Animal 10.1		Animal 10.2		Animal 10.3		Animal 10.5		Animal 10.6		Animal 10.7	
Time.	Reaction time.	Time.	Reaction time.	Time.	Reaction time.	Time.	Reaction time.	Time.	Reaction time.	Time.	Reaction time.
	sec.		sec.		sec.		sec.		sec.		sec.
12.01	1.3	12.03	1.4	12.04	1.3	12.07	1.5	12.09	1.4	12.10	1.3
5.29	1.3	5.30	1.4	5.31	1.2	5.34	1.4	5.35	1.4	5.37	1.5
10.27	1.3	10.28	1.3	10.30	1.5	10.33	1.5	10.34	1.5	10.35	1.3

termination several times on one animal. This possesses the advantages of convenience and greater numbers.

2. The reaction time of *Mya* is similar to that of *Ciona* in respect to its composition. It may be divided into two periods. The first is a sensitization period during which *Mya* must be exposed to the stimulating light in order to respond with the usual reaction time. The second interval is a latent period during which the presence or absence of the stimulating light in no way influences the duration of the reaction time. During this latent period *Mya* may be in the dark, but will respond as usual to the effect initiated by the light during the sensitization period.

The composition of the reaction time in *Mya* is striking because of the disparity in the duration of its two phases. The latent period may vary between 1 and 4 seconds depending on the temperature.

At a temperature of 20°C. it is approximately 1.3 seconds. The sensitization period, however, is very small, resembling in duration the exposure periods associated with photographic processes.

The best demonstration of the composition of the reaction time is, in fact, by the use of a photographic shutter. An animal is kept in a dark room, and observations are made with the aid of a ruby lamp to which *Mya* is comparatively insensitive. By means of a camera shutter the animal is exposed to light for very short intervals, and its reaction time noted. It was found that with an illumination of 2,000 meter candles, an exposure of $\frac{1}{100}$ second was

TABLE II.

Intensity. (Meter candles.)	Animal 10.6		Animal 10.7	
	Time.	Reaction time.	Time.	Reaction time.
		sec.		*sec.*
435	12.27	1.3	12.28	1.3
	4.25	1.4	4.27	1.2
	9.12	1.3	9.13	1.0
690	2.13	1.2	2.14	1.2
	5.35	1.4	5.37	1.5
	10.53	1.5	10.54	1.2
895	1.37	1.3	1.39	1.2
	5.11	1.3	5.13	1.3
	10.05	1.3	10.06	1.4

more than enough to cause a response of the animals in the usual reaction time.

3. Within a moderate range of intensities, and at a constant temperature, the latent period remains the same. This may be demonstrated by determining the reaction time of an animal to a series of intensities which require sensitization periods of less than 0.1 second. Under such conditions, the sensitization period and its variations due to the changes in intensity will not be noticeable in the reaction time as measured with a stop-watch. Table II records the reaction time of two animals exposed to three intensities.

It will be seen that the variations in the reaction time, and consequently of the latent period, are well within those of the experi-

mental error. The average of these two sets of determinations is 1.29 seconds, practically all of which represents latent period. At lower intensities, of course, the sensitization period becomes larger, and enters perceptibly into the duration of the reaction time as ordinarily measured.

4. The constancy of the latent period under given conditions of temperature and intensity is of prime significance in the study of dark adaptation. Because of its uniformity, any variations in the reaction time under these conditions signify variations not in the latent period, but in the sensitization period.

The sensitization period represents the time required for the light to produce its chemical effect. Any variation in its duration means a change in the amount of chemical effect. In terms of the photochemical law of Bunsen and Roscoe,—which has been shown to apply to such sensitization processes (Loeb, 1918, Hecht, 1918, *b*),—the amount of photochemical change at a given intensity varies with the time of exposure to light. For such short periods as occur here it may be safely assumed that the photochemical effect is a linear function of the time of exposure to a given intensity. The duration of the sensitization period may thus be taken to represent the amount of photosensitive substance decomposed in the sense organ by the action of the light. Therefore determinations of the reaction time at constant temperature and intensity furnish us with a measure of the amount of chemical effect necessary to produce a response during various phases of light and dark adaptation in *Mya*.

Photic Adaptation.

1. The exposure of *Mya* to light results in only a single response. After retracting its siphons, the animal slowly expands them, and remains in this state during the remainder of the exposure. This acquisition of sensory equilibrium was accomplished at all the intensities to which *Mya* was subjected, the highest intensity used being 10,000 meter candles.

If, after such an adaptation, *Mya* is placed at a lower intensity, or preferably in the dark, it rapidly becomes sensitive to the light with which it had been in sensory equilibrium. By determining

the reaction time at frequent intervals, it is possible to follow the progress of the acquisition of sensitivity.

The details of an experiment are as follows. An animal is exposed to an intensity of about 3,000 meter candles. At the end of an hour the light is turned off and the animal left in complete darkness except for the presence of a ruby light. After definite intervals the response of the animal to a light of 225 meter candles is determined by recording the reaction time.

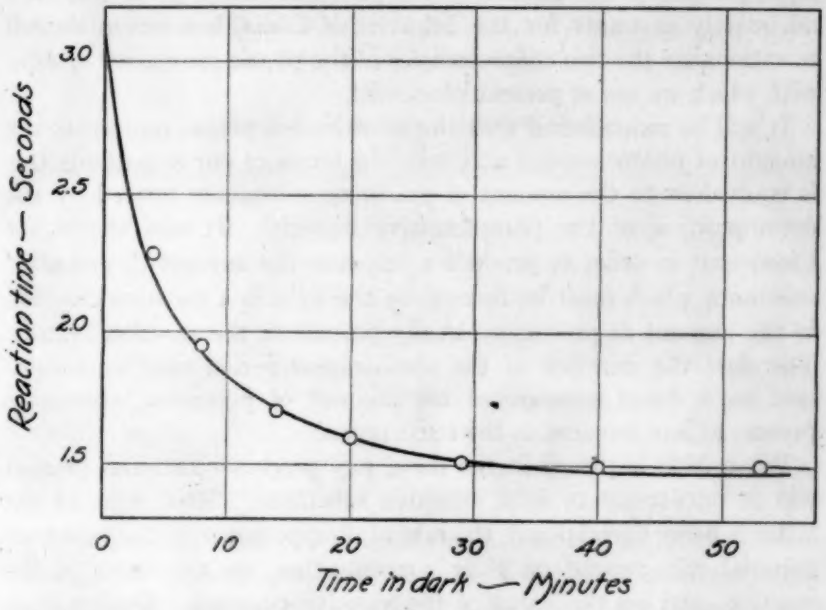

FIG. 1. The course of dark adaptation. Each point is the average of six determinations of the reaction time. The curve represents the progress of a chemical reaction of the second order with a velocity coefficient of $k = 0.245$.

Preliminary experiments showed that the animals became sensitive to the weak light in less than 5 minutes. From then on the reaction time became progressively shorter until it reached a constant minimum in about 45 minutes. Six experiments were therefore made on as many animals, under conditions of exposure, temperature, etc., as similar as possible. The data are given in Fig. 1. Each point represents the average of six determinations. It is apparent

from the position of the points that the progress of dark adaptation in *Mya* follows a decidedly regular course.

2. The hypothesis which was suggested to explain the photic responses of *Ciona* (Hecht, 1918, *b*), involves the existence of a reversible reaction sensitive to light. The action of light is to decompose a photosensitive substance into its precursors. The recombination of the precursor substances to form sensitive material is, however, independent of the presence of light, and proceeds whenever any fresh precursors are formed. This photosensitive system not only adequately accounts for the behavior of *Ciona*, but serves as well to rationalize the two characteristics of the photic sensitivity of *Mya* with which we are at present concerned.

It will be remembered that the sensitization period represents the amount of photochemical activity. In terms of our hypothesis this is equivalent to the amount of precursor substances formed by the decomposition of the photosensitive material. It was shown for *Ciona* that in order to produce a response the amount of precursor substance which must be formed by the light is a constant fraction of the amount of precursor already present in the reaction system. Therefore the duration of the sensitization period may be considered as a direct measure of the amount of precursor substances present at any moment in the sense organ.

When *Mya* is placed in the dark, any precursor material present will at once react to form sensitive substance. Since none of the latter is being decomposed, the rate of disappearance of the precursor material will depend on their concentration, on the order of the reaction, and on the value of the velocity constant. During dark adaptation then, the change in the sensitization period should reveal the rate of formation of photosensitive substance.

In view of this reasoning it is highly significant that the curve drawn in Fig. 1 is an isotherm of a bimolecular reaction.

That the points fit the theoretical curve is apparent from mere inspection. However, in order to bring this out in the customary way, Table III is presented, giving the average observed values for the reaction time and those calculated according to

$$k = \frac{1}{at} \cdot \frac{x}{a-x}$$

the equation for a bimolecular reaction.

In the equation, k equals 0.245 at this temperature of 22°C.; a is the amount of precursor material present at the first moment of darkness (equal to 100 per cent); x is the per cent of photosensitive substance formed in the time t; and $a-x$ is the amount of precursor substances still remaining.

In making the calculations, the sensitization period corresponds to $a-x$, and a is its value when $t = o$, in this case 1.79 seconds. In addition it must be remembered that the reaction time represents not only the sensitization period, but also, at this temperature, a latent period of 1.31 seconds.

TABLE III.

Reaction Time During Dark Adaptation.

$k = 0.245$

t	Reaction time.	
	Observed.	Calculated.
min.	*sec.*	*sec.*
4	2.28	2.21
8	1.95	1.92
14	1.70	1.71
20	1.60	1.60
29	1.51	1.53
40	1.50	1.48
53	1.50	1.47

As a result of these experiments on dark adaptation it is therefore possible to express quantitatively the hypothesis previously suggested. The photosensitive system resolves itself into three components concerned in the two reactions of a reversible system. The first reaction is independent of light and consists in the combination of *two* precursor substances (P and A) to form a photosensitive material (S). The second reaction involves the decomposition of S into its two precursors. The system may therefore be written

$$S \underset{\text{"dark"}}{\overset{\text{light}}{\rightleftharpoons}} P + A$$

with a full consciousness of the quantitative significance of the expression.

3. The dynamics of this reversible reaction must explain the peculiarities of photosensory adaptation. The course of dark adaptation is, of course, clearly a function of the unopposed "dark" reaction. In addition, however, it must be shown how the hypothesis accounts for sensory equilibrium, and for the difference in sensitivity of light- and dark-adapted sense organs.

In the presence of any intensity of light the two opposing reactions will presently reach a stationary condition, at which the concentration of the three components will remain constant. This is equivalent to sensory equilibrium, and involves two characteristics. One is that this intensity no longer acts as a stimulus. This follows from the fact that no increase in the amount of precursor substances takes place. The second is that from this level the reaction time to a higher intensity is always the same. This is because a response always involves the formation of the same fraction of the amount of precursor substances present in the system. Since the latter is constant, the reaction time will also be constant.

The reaction system may approach a stationary condition from either side and at different intensities (Sheppard, 1914). Similarly, sensory equilibrium may be attained either at low intensities through dark adaptation, or at high intensities through light adaptation. In the reaction system a low intensity means a small quantity of precursor substances present at the stationary condition, whereas at high intensities a large quantity will be present. This is true of reversible photochemical reactions, as well as of those that are only pseudoreversible (Sheppard, 1914, p. 225). In order to produce a response the amount of precursor formed must be a definite fraction of the amount then present in the system. Hence at a high intensity sensory equilibrium more light energy will be required to produce a response than at a low intensity equilibrium. This is equivalent to saying that a dark-adapted animal is more sensitive to light than a light-adapted one,—which is a self-evident proposition of sensory physiology (von Helmholtz, 1911, Hess, 1910).

The photochemical system here proposed is therefore adequate to account for the phenomena associated with the sensory adaptation of *Mya* in its responses to an increase of illumination.

Effect of Temperature.

1. The significance of an hypothesis is materially strengthened by the testing of certain corollaries that follow from its acceptance. One such prediction was investigated in the case of *Ciona* with results that amply justified the application of the hypothesis. The greater definiteness of the reaction system in the case of *Mya* made possible the investigation of other phases of the hypothesis.

It is well known that the rate of photochemical reactions is comparatively uninfluenced by a change in the temperature (Redfield and Bright, 1919). Therefore the decomposition of photosensitive material, $S \rightarrow P + A$, should proceed practically independent of the temperature. The reverse reaction, however, $P + A \rightarrow S$, being an ordinary bimolecular reaction and independent of the presence of light, should be decidedly influenced by the temperature.

Two things obviously follow from these conceptions. The first is that the amount of precursor substances present at the "stationary state" for a given intensity will vary inversely with the temperature. The rate of formation of precursor substances being practically the same at all temperatures, the amount of precursor substances present will depend solely on the rate of their recombination. Therefore at low temperatures there will be more precursor in the system than at high temperatures. Since the sensitization period at any moment measures the amount of precursor substances present in the system, the sensitization period for a sensory equilibrium at a given intensity will vary inversely with the temperature.

The second corollary concerns the rate of dark adaptation. This is determined entirely by the reaction $A + P \rightarrow S$. With the complete exclusion of light, this reaction should proceed faster at higher temperatures and slower at lower temperatures. The curve of dark adaptation as given in Fig. 1 should therefore show changes with temperature of the kind usually associated with chemical reactions.

2. Both of these points were investigated, and the experiments verified the assumptions unequivocally. The procedure was essentially the one previously described for the experiments on dark adaptation. After determining the rate of dark adaptation at room temperature on an animal, it was kept undisturbed for several hours

in the dark. After such a rest, the experiment was repeated at a lower temperature. This means that both the exposure to the intense light and the dark adaptation were conducted at the lower temperature. After some hours of rest the experiment was again repeated at a still lower temperature.

Mya is a particularly good animal for such purposes because the continuous water current from its siphons goes through the body and serves to stir the water. The animal is thus maintained at the temperature of the water. Reliance was, however, not placed upon this, and the usual precautions were taken for maintaining an approximately constant temperature throughout an experiment.

The results obtained with five animals are shown in Fig. 2. Each point represents an *individual* reading of the reaction time of the animal indicated. The data as plotted serve, therefore, to show the experimental error as well as the regularities observed. In order to point out the trend of the data of Fig. 2, I have drawn as solid lines the course of a reaction of the second order at the three temperatures indicated. The velocity coefficients (k) are 0.095 at 11.5°C., 0.182 at 16.2°C., and 0.228 at 21.9°C. In making the calculations according to the equation

$$k = \frac{1}{at} \cdot \frac{x}{a-x}$$

it should be remembered here also that the latent period is to be subtracted from the reaction time to get the sensitization period. The latent period varies with the temperature (Hecht, 1919). For this group of animals and at the temperatures indicated above, the latent periods are, respectively, 2.02, 1.57, and 1.31 seconds. How well the theoretical curve corresponds with the observed individual readings is clear from the figure. Table IV in addition gives a comparison between the calculated and the average observed values of the reaction time.

The data show the two corollaries of the hypothesis to be correct. Taking the value of $a-x$ at $t = o$ we get the sensitization period for the last moment of sensory equilibrium at the three temperatures. These are 5.38 seconds at 11.5°, 3.43 seconds at 16.2°, and 1.79 seconds at 21.9°. They therefore show a decided increase

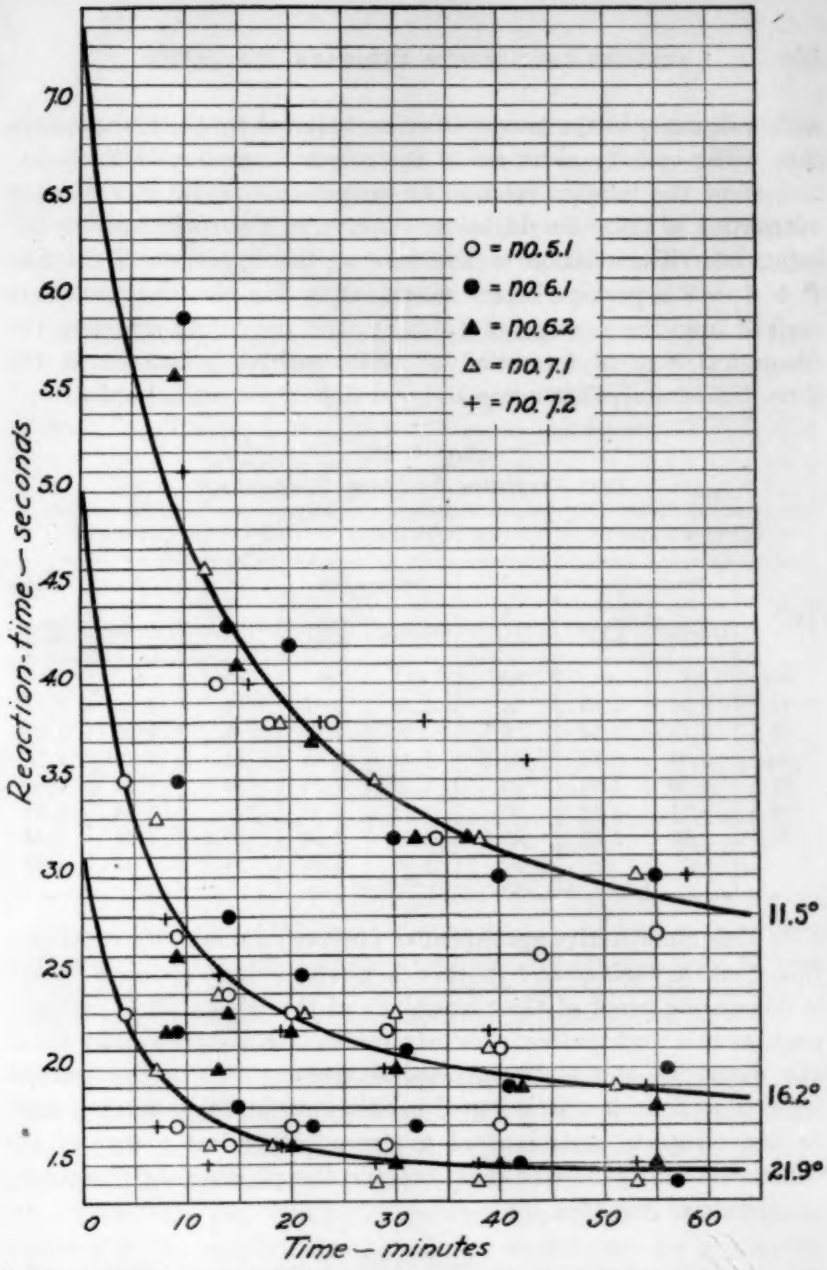

FIG. 2. The course of dark adaptation at three different temperatures. The points are the individual measurements of the reaction time of the five animals indicated in the legend. The curves are those of a bimolecular reaction at the different temperatures. Their velocity coefficients are given in the text.

with a decrease in the temperature, as required by the first assumption. The velocity constants of the curves drawn for dark adaptation show the familiar relation to temperature. The rate of dark adaptation is faster for higher temperatures, the coefficient for 10° being 2.4. The relation of the data to the hypothetical reaction $P + A \rightarrow S$ is perhaps better illustrated by Fig. 3. The points are derived from the averages of Table IV, and the curves represent the theoretical rate of formation of photosensitive substance at the three temperatures according to a reaction of the second order.

TABLE IV.

Dark Adaptation at Different Temperatures.

11.5°C.; $k = 0.095$			16.2°C.; $k = 0.182$			21.9°C.; $k = 0.228$		
t	Reaction time.		t	Reaction time.		t	Reaction time.	
	Observed.	Calculated.		Observed.	Calculated.		Observed.	Calculated.
min.	*sec.*	*sec.*	*min.*	*sec.*	*sec.*	*min.*	*sec.*	*sec.*
11.	5.04	4.68	4.	3.50	3.55	4.	2.30	2.25
15.	4.13	4.24	8.	2.98	2.94	8.	1.98	1.94
20.	3.94	3.86	13.5	2.44	2.56	13.	1.70	1.76
29.5	3.50	3.44	20.	2.30	2.30	19.5	1.64	1.64
39.5	3.11	3.13	30.	2.12	2.10	29.	1.54	1.54
55.	2.92	2.89	40.5	2.05	2.00	39.5	1.52	1.49
			54.	1.90	1.89	54.5	1.45	1.44

3. This quantitative agreement of observed values with those predicted on the basis of an hypothetical photosensitive chemical system is convincing proof of the plausibility of the latter. It is my purpose, as this work proceeds, to map out in increasingly greater detail the dynamics of this photochemical system, and to test various deductions from it. It is hoped in this way finally to reach a more or less complete statement of the physicochemical nature of the substances and processes that underlie the photosensory responses of an animal like *Mya*.

SUMMARY.

1. The reaction time of *Mya* to light is composed of two parts. The first, a sensitization period, is an exceedingly short interval of

the order of magnitude associated with photographic processes. The second is a latent period of about 1.3 seconds, during which *Mya* need not remain exposed to the stimulating light.

2. The process of dark adaptation in *Mya* is orderly. Its progress may be represented by the formation of a photosensitive substance according to the dynamics of a bimolecular reaction.

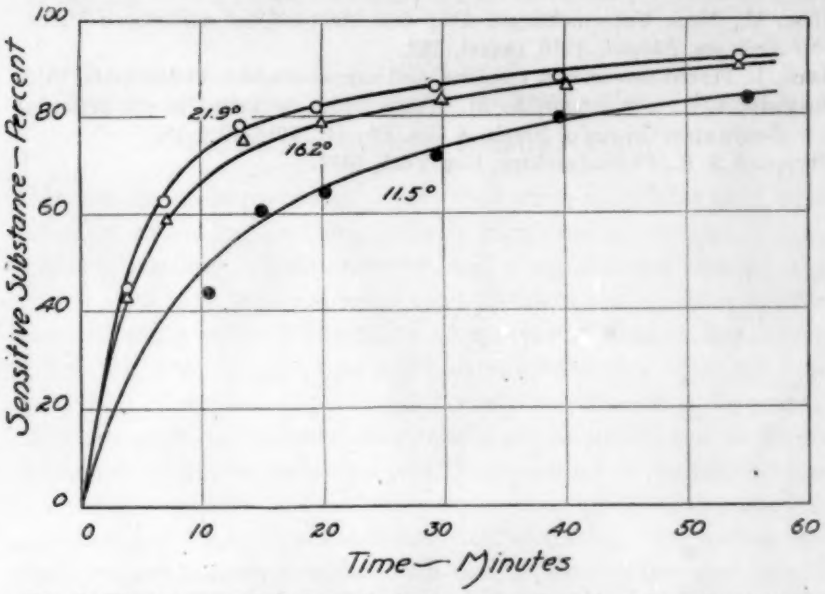

FIG. 3. The progress of the hypothetical reaction, $P + A \rightarrow S$, at the temperatures indicated. The points are calculated from the averages given in Table IV, and represent the amount of photosensitive material formed at different stages of dark adaptation. The solid lines give the theoretical values.

3. Photosensory equilibrium as represented by the light- and dark-adapted conditions finds a rational explanation in terms of the "stationary state" of a reversible photochemical reaction involving a photosensitive substance and its two precursors.

4. There are two corollaries to this hypothesis. The first requires that the reaction time at sensory equilibrium for a given intensity should vary inversely with the temperature; the second, that the rate of dark adaptation should vary directly with the temperature. Experiments verified both of these requirements.

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AMPHOTERIC COLLOIDS.

V. The Influence of the Valency of Anions upon the Physical Properties of Gelatin.

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(Received for publication, February 21, 1919.)

It was shown in preceding papers that while all gelatin salts with univalent cation show a comparatively high osmotic pressure, a high degree of swelling, a high viscosity, and a high alcohol number, the gelatin salts with bivalent metal (and trivalent metal) show for the same pH and gelatin concentration a low osmotic pressure, low degree of swelling, low viscosity, and a low alcohol number. Since the conductivities of the two types of gelatin salts were found to be practically the same, the valency effect could not be attributed to differences in the degree of ionization and "hydratation" of the gelatin ions. The writer suggested as a tentative explanation the assumption of an aggregate formation of a stoichiometrical character. He had shown that two gelatin ions combine with one bivalent cation and it was suggested that in the process of electrolytic dissociation these two gelatin ions form one anion with two charges. This suggestion is able to explain quantitatively the results actually observed.[1]

In one of his previous publications the writer had already called attention to the fact that bivalent anions differ in their valency effect from bivalent cations.[2] While SO_4 has a similar depressing effect upon the physical properties of gelatin as Ca or Ba, the same does not seem to be true for other bivalent and trivalent anions like oxalate, succinate, tartrate, citrate, or phosphate. The acids of these latter anions seemed to act as if they were monobasic acids, one molecule of these acids being capable of combining with one gelatin ion only.

[1] Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 483.

[2] Loeb, J., *J. Biol. Chem.*, 1918, xxxiv, 489.

It seemed very important to test this possibility, since a decision might serve as a crucial test for or against our purely chemical conception of the behavior of colloids, according to which the effect of an electrolyte upon the physical properties of the colloid is due to the formation of real chemical compounds between the colloid and one of the ions of the electrolyte. If it can be shown that SO_4 has a similar depressing effect as a bivalent cation we must also be able to prove that SO_4 combines with two molecules of gelatin; and if it can be shown that PO_4, citrate, oxalate, succinate, etc., have no such depressing effect upon the physical properties of gelatin but behave like Br, Cl, NO_3, and acetate, we must also be able to prove that one molecule of each of these acids combines with only one molecule of gelatin.

We are able to decide this question in favor of the purely chemical theory of colloids.

The decision was rendered possible through the proof that we can ascertain the amount of acid in combination with a given mass of gelatin by titration with NaOH.[3] If we treat gelatin with acid and titrate afterwards with NaOH, we obtain the mass of acid in combination with the gelatin with the aid of two corrections; namely, first by deducting from the titration value a constant value which is 1.8 cc. 0.01 N NaOH for 10 cc. of a 1 per cent gelatin solution, titrating to neutrality (pH = 7.0). This constant value 1.8 cc. 0.01 N NaOH is the quantity of alkali required to bring 0.1 gm. isoelectric gelatin to pH 7.0. The correctness of this method was demonstrated in the case of HBr and HCl in which it was possible to compare the amount of NaOH required for the titration of a 1 per cent gelatin solution with the results of volumetric analysis for Br according to Volhard's methods. It was found that the Br (or Cl) numbers agreed with the NaOH numbers when the constant value mentioned above was deducted. In that paper[3] we had titrated for pH = 9.0, while in the experiments reported in the present paper we titrate for pH = 7.0; as a consequence the value of the constant given in the first paper was higher than 1.8 cc. 0.01 N NaOH.

[3] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 363.

In addition a second correction is needed as explained in a previous paper. The gelatin treated with acid contains some free acid, the titration value of which has also to be deducted from the titration number. This correction can be found by titration of the same acid at the same pH but free from gelatin.

With the aid of these two corrections we are also able to investigate the effect of acid on gelatin in the presence of the acid. It was observed that the maximal osmotic pressure of gelatin chloride or sulfate is reached at a pH of between 3.3 to 3.5. This maximum is never reached when we wash the gelatin four or six times with H_2O after a treatment with an acid of moderate concentration, as was done in our previous experiments, since in this washing the acid formed by hydrolytic dissociation is removed, which causes new hydrolysis and in this way in successive washings a rapid decline of the pH results, as will be shown more definitely in a subsequent paper.

Since it was necessary for our purpose to be always sure of ascertaining the maximal osmotic pressure a 1 per cent solution of isoelectric gelatin can reach when treated with different acids, we were forced to adopt a different method from that described in the preceding papers. We melted 1 gm. of gelatin rendered isoelectric in the manner described in a previous paper and then added different quantities of 0.01 N or 0.1 N acid and made up the volume to 100 cc.[4] This solution was put into a collodion bag of a capacity of about 50 cc. and the bag was put into a beaker containing 400 cc. of the same percentage of acid in distilled water as that added to the gelatin solution. The collodion bag was closed tightly with a perforated rubber stopper containing a glass tube serving as a manometer to measure the osmotic pressure of the gelatin solution. After about 18 hours, when osmotic equilibrium was reached, the pH, the conductivities, and the titration numbers of the acid inside and outside the bag were determined, as were also the osmotic pressures.

[4] For the sake of briefness we shall designate such solutions as 1 per cent gelatin solutions, although this does not take into account the increase in weight of the gelatin due to the combination with another ion.

Table I gives the data of one experiment with a 1 per cent solution of isoelectric gelatin made up in 100 cc. of H_2O containing varying quantities of 0.01 N HBr, stated in the upper horizontal row. The second horizontal row of figures gives the pH of the gelatin solution at the end of the experiment, and the third row the figures for the cc. of 0.01 N Br found by Volhard's method, in 10 cc. of the gelatin solution. These latter figures are too high since they include the free HBr in the solution. We can find the value for the free HBr solution from the pH and this value must be deducted from the titration number for Br. This deduction gives us the corrected Br values of the horizontal Row 4. Row 5 gives the number of cc. of 0.01 N HBr found in 10 cc. of the gelatin solution by titrating with NaOH to pH = 7.0. This value demands, as stated, two corrections; first, a deduction of the amount of NaOH needed to bring 10 cc of 1 per cent isoelectric gelatin solution to pH = 7.0. This value is 1.8 cc. of 0.01 N NaOH for 10 cc. of a 1 per cent solution of gelatin. The second correction is that for the free HBr present in the gelatin solution which can be measured by titrating free HBr of the same pH as that of the gelatin solution to pH = 7.0. Row 6 gives the titration values for NaOH after the two corrections have been made. These corrected figures should give us the amount of HBr in combination with 0.1 gm. of gelatin. The reader will notice that the corrected numbers for the direct titration for Br after Volhard (Row 4), and the corrected numbers for titration with NaOH (Row 6), are, within the limits of the accuracy of our method, identical, as they should be. This identity is of the greatest importance for the subject we intend to discuss in this paper, since in the case of other acids (with the exception of HCl and H_3PO_4) we can ascertain the amount of acid in combination with 0.1 gm. of gelatin only indirectly by titrating with NaOH. It was, therefore, necessary for us to be sure that this titration gives us the correct amount of acid in combination with 0.1 gm. of gelatin if we make the two corrections mentioned, and the identity of the titration numbers by the direct Volhard method for Br and by the indirect method of titrating with NaOH gives us this certainty.

Row 7 gives the osmotic pressure in mm. of the height of a column of the 1 per cent gelatin solution. Row 8 gives us the conductivity

TABLE I.

1 per cent solution of isoelectric gelatin made up in 100 cc. H_2O containing varying quantities of 0.01 N HBr.																
1. Cc. 0.01 N HBr added	50	40	35	30	25	22.5	20	17.5	15	12.5	10	7.5	5	2.5	1	0.5
2. pH of gelatin solution	2.8	3.0	3.1	3.15	3.25	3.3	3.35	3.4	3.5	3.6	3.75	3.9	4.1	4.3	4.5	4.7
3. Cc. 0.01 N Br found in 10 cc. of gelatin solution	8.55	7.3	6.8	6.4	5.7	5.4	4.85	4.45	4 0	3.3	3.0	2.4	1.5	0.65	0.2	0.1
4. Corrected values of Br	7.0	6.1	5.8	5.6	5.1	4.9	4.5	4.05	3.66	3.05	2.8	2.28	1:42	0.55	0.15	0
5. Cc. 0.01 N NaOH required to bring 10 cc. of gelatin solution to pH 7.0	10 0	9.2	8.4	8.0	7.3	7.0	6.55	6.3	5.8	5.15	4.7	4.2	3.5	2.7	2.15	1.9
6. Corrected NaOH values	6.7	6.25	5.6	5.4	4.9	4.7	4.35	4.1	3.66	3.1	2.7	2.28	1.62	0.8	0.3	0
7. Osmotic pressure in mm. of height of column of a 1 per cent gelatin solution	188	230	256	292	294	310	306	330	330	305	292	256	204	100	45	26
8. Conductivity $\left(\frac{10{,}000}{\text{ohms}}\right)$, corrected for pH		21.2	18.85	16.8	14.9		13.1	11.1	9.7		7.2			1.47	0.51	
Measurements of pure acid solution (free from gelatin) in the outside beaker.																
9. pH of acid solution	2.2	2.3	2.35	2.4	2.5	2.6	2.75	2.9	3.0	3.1	3.1	3.25	3.35	3.8	4.3	4.5
10. Br number	4.85	3.6	3.15	2.7	2.3	1.9	1.7	1.5	1.2	1.0	0.8	0.6	0.35	0.2	0.15	0.05
11. NaOH number	4.7	3.6	3.0	2.6	2.1	1.9	1.65	1.45	1.15	1.0	0.8	0.6	0.4	0.2	0.1	0.05
12. Conductivity $\left(\frac{10{,}000}{\text{ohms}}\right)$	54.6	42.8	37.0	32.8	25.6	22.3	20.0	17.1	13.8	11.35	9.0	6.67	4.07	1.86	0.86	0.48

$\left(\frac{10{,}000}{\text{ohms}}\right)$ corrected for the pH (*i.e.*, after deduction of the conductivity of a free acid solution with the pH found in the gelatin solution).

Rows 9 to 12 give the measurements of the pure acid solution (free from gelatin) surrounding the collodion bag containing the gelatin solution. The pH in the outside solution is always lower than inside the bag and this difference may find its explanation on the basis of the theory of equilibrium developed by Donnan for such cases. The values for titration for Br by the Volhard method and for the titration for HBr by titration with NaOH are practically identical for this pure acid solution, thus showing that the degree of accuracy of the method of titration was adequate.

In Table II the measurements for 1 per cent isoelectric gelatin treated with H_2SO_4 are given. Here we can ascertain the amount of SO_4 in combination with 0.1 gm. of gelatin only indirectly by titrating with NaOH, applying the two corrections mentioned. All the other measurements are the same as in the experiment with HBr.

A comparison of the corrected NaOH values in Row 4, Table II, with those in Row 6, Table I, shows that they are practically identical for the same pH. Thus for pH = 3.35 the corrected NaOH value is 4.4 in the case of gelatin sulfate, and 4.35 for gelatin bromide. Since the experiments with HBr have shown that these corrected NaOH values are identical with the Br values we can state, that for a given pH 1 gm. of gelatin is in combination with twice as many atoms of Br as with radicles of SO_4; or in other words, that H_2SO_4 behaves toward gelatin as a dibasic acid. Fig. 1 illustrates this identity of the curves for NaOH values of the two acids. In spite of the identity of equivalents of these two ions in combination with 1 gm. of gelatin the osmotic pressures are very different for the two types of gelatin salts. This is illustrated by Fig. 2, where the ordinates represent the osmotic pressures and the pH are the abscissæ. It is obvious that the values of the osmotic pressure of gelatin bromide are considerably higher than the values for the osmotic pressure of gelatin sulfate. The reader will notice that the maximum osmotic pressure for gelatin bromide is about 325 mm., which is identical with the maximal osmotic pressure found for gelatin salts with univalent cation, *e.g.* sodium gelatinate; while the maximum value for gelatin sulfate is about 130, almost identical with the maximum for calcium

TABLE II.

1 per cent solution of isoelectric gelatin made up in 100 cc. H_2O containing varying quantities of M/100 H_2SO_4.

1. Cc. M/100 H_2SO_4	45	40	35	30	25	22.5	20	17.5	15	12.5	10	7.5	5	2.5	0
2. pH of gelatin solution	2.4	2.45	2.5	2.65	2.75	2.85	2.9	2.95	3.0	3.1	3.2	3.35	3.55	4.1	4.8
3. Cc. 0.01 N NaOH required to bring 10 cc. of gelatin solution to pH 7.0	15.2	14.4	13.8	12.5	11.6	10.9	10.0	10.4	9.1	8.3	7.8	6.6	5.5	3.8	1.8
4. Corrected NaOH values	9.55	9.2	9.0	8.9	8.2		6.7		6.2	5.7	5.4	4.4	3.4	1.85	0
5. Osmotic pressure in mm. of height of column of a 1 per cent gelatin solution	63	74	77	80	87	90	91	92	114	123	129	132		112	34
6. Conductivity $\left(\frac{10{,}000}{\text{ohms}}\right)$, corrected for pH	31.0		30.1		26.0		18.1		15.25	10.85		9.8	7.3	3.95	0.52

Measurements of pure acid solution (free from gelatin) in the outside beaker.

7. pH of acid solution	2.1	2.2	2.2	2.3	2.35	2.4	2.45	2.5	2.55	2.65	2.75	3.0	3.3	3.5	5.0
8. NaOH number	8.0	7.3	6.4	5.2	4.4	3.85	3.4	3.0	2.4	1.8	1.4	1.1	0.7	0.3	
9. Conductivity $\left(\frac{10{,}000}{\text{ohms}}\right)$	81.3	74.0	65.8	55.3	47.6	47.9	35.5	33.2	27.4	21.4	16.1	11.75	7.46	3.22	0.22

gelatinate. The maxima for both gelatin bromide and gelatin sulfate are found at the same pH, namely between 3.3 and 3.5. van't Hoff's theory would ascribe the difference in the maximal osmotic pressures of the two acids to a corresponding difference in the number of particles in solution; while the colloid chemists would ascribe the difference in osmotic pressure to a difference in the "hydratation" due to a difference in the ionization of the two gelatin salts.

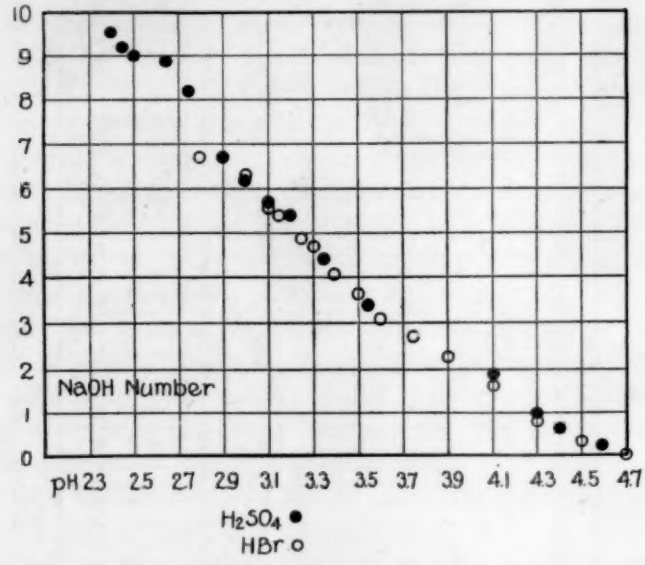

FIG. 1. Relative amount of HBr and H_2SO_4 in combination with gelatin Abscissæ represent pH; ordinates, amount of 0.01 N HBr and H_2SO_4 bound by 0.1 gm. of gelatin. The identity of the curves for the two acids proves that H_2SO_4 combines with gelatin as a dibasic acid.

The curves for conductivity of the two gelatin salts (plotted according to the corrected conductivity values of Table I and Table II) differ but slightly (Fig. 3). As a matter of fact, if other experiments with the two acids are taken into account (one of which will be given later) the curves for the conductivities of the two gelatin salts are practically identical if plotted over pH as abscissæ. This identity of the conductivity curves contradicts the hydratation hypothesis of the colloid chemists.

We therefore are confronted with the same situation as in the case of sodium gelatinate and calcium gelatinate, where we also found practically equal conductivity combined with a ratio of osmotic pressures of 3:1 for the two metal gelatinates mentioned. In the latter case we offered the tentative assumption that the Ca ion combines

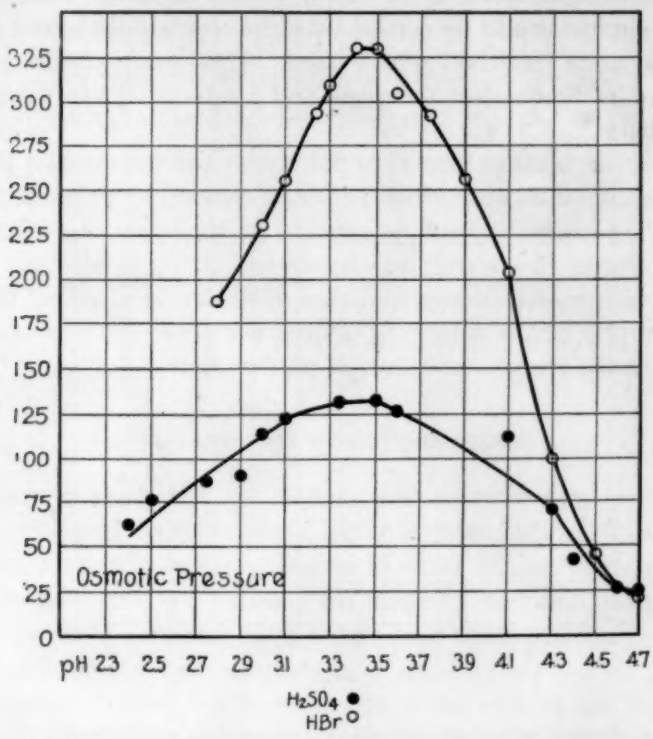

FIG. 2. Osmotic pressure curves for gelatin sulfate and gelatin bromide. Abscissæ represent pH; ordinates, osmotic pressure, showing that for the same pH the osmotic pressure is higher when HBr than when H_2SO_4 is added to gelatin. The maximal osmotic pressure for both acids is found at the same pH (about 3.5).

with two gelatin anions, these two gelatin anions remaining together in a single aggregate when electrolytic dissociation occurs. If the compound Ca_2 gelatin$_4$ dissociates into two Ca ions and one aggregate of four gelatin anions carrying four charges, the same number of charges, namely eight, would be carried by the dissociation of four

sodium gelatinate molecules yielding eight charges and eight ions. This would produce equality of charges and a ratio of osmotic pressures of 3:8. The same assumption applied to gelatin bromide and gelatin sulfate would demand that $gelatin_4(SO_4)_2$ dissociate into three particles, two negative SO_4 ions and one aggregate ($gelatin_4$) with four positive charges; while in the case of gelatin bromide the same number of charges would be carried by eight separate ions, four Br and four separate positive gelatin ions. This would again produce equality in the number of charges and a ratio of 3:8 in the number of particles.

When pH becomes less than 3.3 the values for osmotic pressure drop again. This drop which had been noticed by previous investigators, especially by Pauli, is ascribed by the latter to a diminution in the degree of electrolytic dissociation of the gelatin salt. This assumption meets, however, with a difficulty in the fact that the conductivity of the gelatin salts does not show this drop but continues to rise steeply for values of pH less than 3.3.

Antagonism between HBr and H_2SO_4.

The real crux between the colloidal and the chemical conception seems to lie in the question of the justification of the assumption of "hydratation" as the cause of osmotic pressure of protein solutions. The writer does not question the possible correctness of the idea that ions are in general surrounded by a jacket of water molecules; he only doubts the correctness of the idea that this possible hydratation of the protein ion is the cause of the osmotic pressure, the swelling, and the other physical properties of proteins as Pauli, Michaelis, and others assume. The following experiment seems to be a further proof against this application of the hydratation hypothesis. We had seen that a 1 per cent solution of both gelatin bromide and gelatin sulfate reaches the maximal osmotic pressure at a pH of about 3.5 and that for this pH the same amount of gelatin is combined with equivalent amounts of Br and SO_4, namely 0.1 gm. of gelatin binding about 3.6 cc. of 0.01 N Br or SO_4. Both solutions have the same conductivity, namely about 9.7. In order to produce gelatin bromide or gelatin sulfate of a pH of about 3.4 it is necessary

that 1 gm. of isoelectric gelatin should be melted and added to 15 cc. of 0.01 N HBr or H_2SO_4 and that the volume be made up to 100 cc. by adding distilled water. By mixing HBr and H_2SO_4 in various

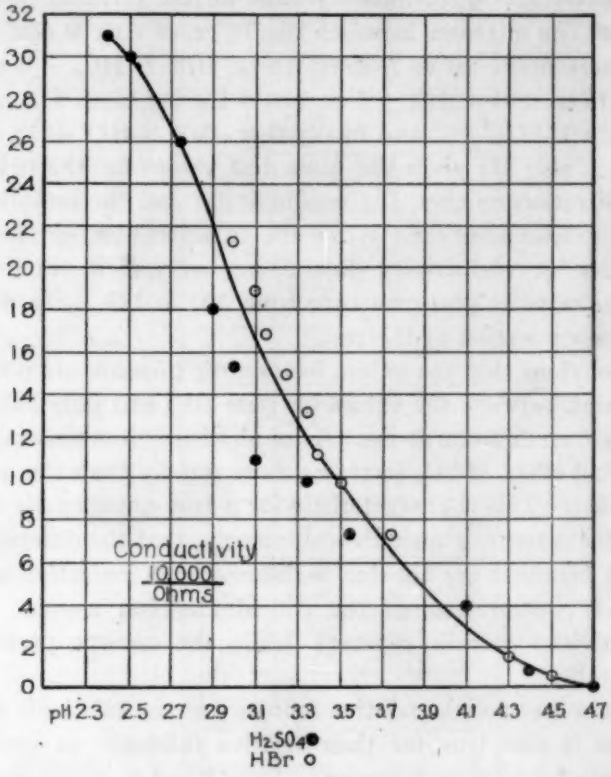

FIG. 3. Conductivity curves for gelatin sulfate and gelatin bromide. Abscissæ represent pH; ordinates, conductivity of gelatin sulfate and gelatin bromide, showing that aside from a few irregular values the curves are practically identical for the two acids. This disproves the assumption that the differences in osmotic pressure, as shown in Fig. 2, can be attributed to differences in ionization and "hydratation" of the two gelatin salts.

proportions but always adding 15 cc. of 0.01 N acid to 1 gm. of isoelectric gelatin, melting the latter, and making up the volume to 100 cc. by the addition of water, the amount of gelatin salt formed and its degree of ionization, *i.e.* the conductivity, should remain the

same, while the osmotic pressure should vary according to the relative proportion of HBr and H_2SO_4 used. HCl was used in this experiment instead of HBr since we had found that HCl and HBr act qualitatively and quantitatively alike on the physical properties of gelatin. The mixtures in which the 15 cc. of 0.01 N acid were prepared were made up as follows: 15 cc. 0.01 N HCl + 0 cc. 0.01 N H_2SO_4; 14 cc. 0.01 N HCl + 1 cc. 0.01 N H_2SO_4; 13 cc. 0.01 N HCl + 2 cc. 0.01 N H_2SO_4, etc., and finally 0 cc. 0.01 N HCl + 15 cc. 0.01 N H_2SO_4. Table III gives the numerical values for the pH, the corrected titration number, the conductivity, and the osmotic pressure of each gelatin solution. While the titration number (Row 2) and the values for conductivity (Row 3) are everywhere practically identical, the osmotic pressures vary from 300 to 128. Fig. 4 gives the graphical expression of the result.

It is obvious that the values for osmotic pressure are not the algebraic mean between the values for pure HCl and pure H_2SO_4. The curve is convex towards the axis of abscissæ, thus showing that the depressing effect of SO_4 increases more rapidly than the quantity of SO_4 added. This is characteristic for a true antagonistic salt effect.

The main result is unequivocal; namely, that the differences in the osmotic pressures are not due to differences in ionization and hydratation of protein ions as the colloid chemists assume, since the conductivities remain constant while the osmotic pressures vary considerably.

What is true for the relative influence of the two acids on osmotic pressure is also true for their relative influence on swelling, and viscosity of gelatin as is shown in Figs. 5 and 6.

Proof that Dibasic and Tribasic Acids (with the Exception of H_2SO_4) Behave Practically like Monobasic Acids toward Gelatin.

Of the other dibasic and tribasic acids which the writer has thus far investigated, namely oxalic, tartaric, succinic, citric, and phosphoric acid, none shows the valency influence. We are able to give the explanation for this peculiar behavior by proving that all these acids react with gelatin practically as if they were monobasic acids.

TABLE III.

1 per cent solution of isoelectric gelatin made up in 100 cc. H_2O containing varying quantities of 0.01 N HCl and H_2SO_4.

Cc. 0.01 N HCl	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
Cc. 0.01 N H_2SO_4	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. pH of gelatin solution	3.4	3.35	3.4	3.35	3.35	3.4	3.35	3.4	3.4	3.35	3.35	3.35	3.35	3.35	3.35	3.35
2. Corrected NaOH values	3.3	3.6	3.6	3.6	3.3	3.5	3.8	3.9	3.7	3.9	3.9	3.8	4.0	4.1	4.1	4.1
3. Conductivity $\left(\frac{10{,}000}{\text{ohms}}\right)$, corrected for pH	9.2	10.4	9.1	9.5	8.4	8.1	9.2	9.2	9.5	9.1	9.5	9.3	9.6	9.3	9 4	9.2
4. Osmotic pressure in mm. of height of column of a 1 per cent gelatin solution	300	282	254	236	210	205	172	185	162	166	151	148	136	134	127	128

We repeated the experiments represented in Tables I and II with a number of other acids, the method of adding acid to gelatin being the same in all cases, though the quantity of acid needed to produce

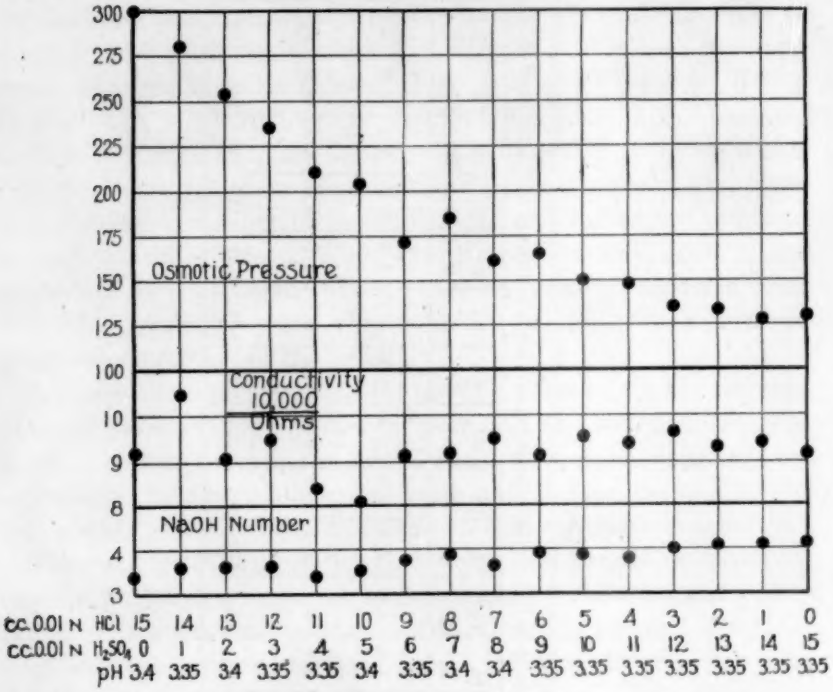

FIG. 4. Effect of mixtures of HCl and H_2SO_4 upon conductivity and osmotic pressure of gelatin. Abscissæ represent mixtures of the two acids; ordinates of lower curve, amount of acid in combination with 0.1 gm. of gelatin. The curve is a straight line, proving that equivalent amounts of H_2SO_4 and HCl are bound by 0.1 gm. of gelatin. Ordinates of middle curve, corrected conductivities of gelatin treated with different proportions of a mixture of the two acids. The curve is also practically a straight line. Upper curve, osmotic pressure curve, showing that the osmotic pressure is the greater the greater the proportion of HCl is in the mixture. The curve is convex to the axis of abscissæ, which is characteristic for antagonism. Notice identity of conductivities and the difference in osmotic pressure, contradicting the hydratation theory of colloid chemists.

the same pH was different for different acids. In the case of phosphoric acid we determined directly (with the uranylacetate method) the PO_4 in combination with 10 cc. of a 1 per cent gelatin solution

treated with different quantities of H_3PO_4. In the case of HNO_3 and oxalic acid we determined the amount of acid in combination with the same amount of gelatin by titration with NaOH for pH = 7.0,

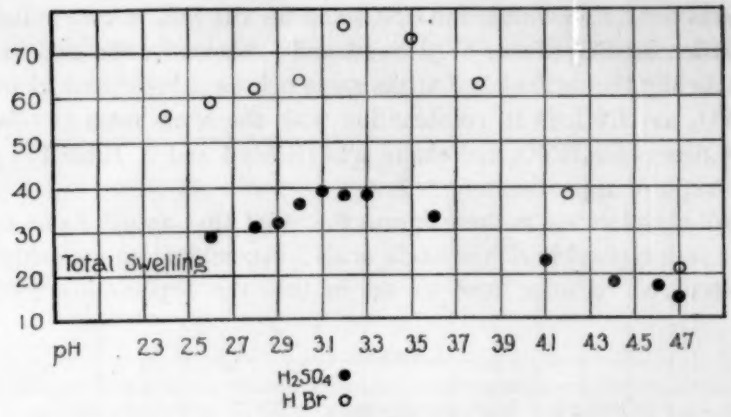

FIG. 5. Relative influence of HBr and H_2SO_4 upon swelling.

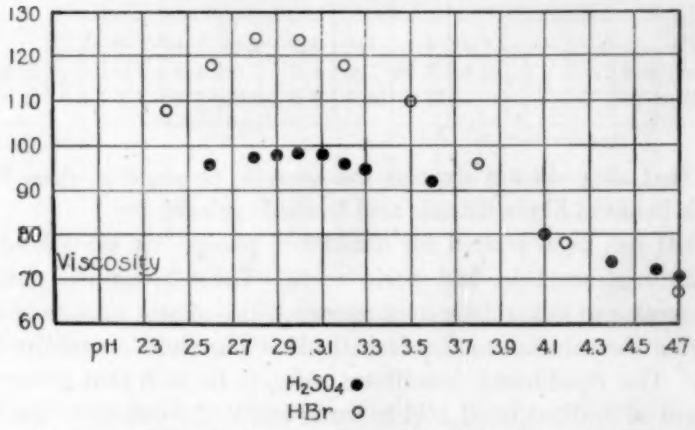

FIG. 6. Relative influence of HBr and H_2SO_4 upon viscosity.

and making the two corrections discussed before. Table IV gives the equivalents of these three acids in combination with 0.1 gm. of gelatin for different pH in terms of cc. of 0.01 N NaOH used for titration.

The values found for HNO_3 are slightly less than those found for HBr and HCl. This harmonizes with the fact that the effect of HNO_3 on the physical properties of gelatin is also slightly less than the effect of HCl or HBr for the same pH. A comparison of the figures for NaOH values for HNO_3 and for the PO_4 values, found by titration for PO_4 (Table IV, Rows 1 and 3) shows for the two values practically the ratio of 1:3 at the same pH; *i.e.*, three times as much H_3PO_4 as HNO_3 is in combination with the same mass of gelatin. The figures for HNO_3 and oxalic acid (Rows 1 and 2, Table IV) give the ratio of approximately 1:2.

We therefore see in these figures the proof that actually one molecule each of phosphoric and oxalic acids is in combination with only one molecule of gelatin; and we see in this the explanation for the

TABLE IV.

Cc. of 0.01 N acid in combination with 10 cc. of a 1 per cent gelatin solution at different pH.

pH	3.1	3.2	3.3	3.4	3.5	3.7	3.9	4.1	4.2	4.3
1. HNO_3	4.35	4.1	3.6	3.2	2.85	2.45	1 9	1.45		0.75
2. Oxalic acid	9.6	8.75	7.6	6.7	6.00	4.3	3.0		1.65	
3. H_3PO_4		12.4	10.4	9.8	9.00	7.4	5.8	4.5	2.6	2.1

fact that they do not depress the osmotic pressure as does H_2SO_4, which behaves like a dibasic acid towards gelatin.

What has been proved for oxalic and phosphoric acids holds also for tartaric, succinic, and citric acids. These latter two acids are very weak and thus relatively large quantities of acid have to be added to bring the solution to the desired pH. The same is true for acetic acid. The equilibrium conditions seem to be such that a very large amount of undissociated acid is found inside the collodion bag and it seems for the present impossible to determine by titration with NaOH the amount of these acids in actual combination with gelatin.

The fact that all the dibasic and tribasic acids mentioned (with the exception of H_2SO_4) behave towards gelatin practically like monobasic acids is a further proof for the theory that the influence of acids on gelatin is determined by the relative amount of gelatin salt formed

through the addition of acid by gelatin. If H_3PO_4 behaves towards gelatin like a monobasic acid the curves for osmotic pressure for gelatin phosphate should be identical with the curves for gelatin nitrate when plotted over pH as abscissæ. Fig. 7 shows that this is actually the case. The maximum for the two curves lies between pH 3.3 and 3.5 and is about 300 mm. Fig. 8 shows that the osmotic pressure curves for gelatin acetate and citrate are also identical and

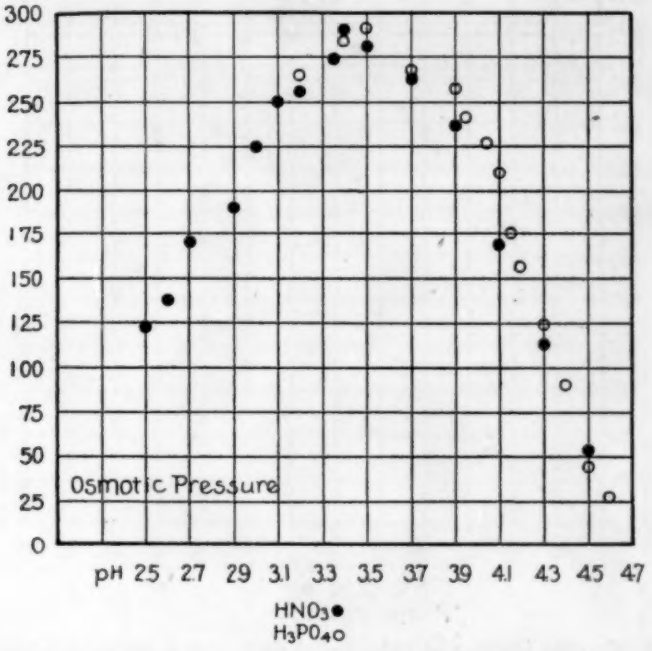

FIG. 7. Showing identity of influence of HNO_3 and H_3PO_4 upon osmotic pressure of gelatin solution for the same pH.

that they agree with the curves for gelatin nitrate and gelatin phosphate as well as with those for gelatin bromide (Fig. 1).

What is true for the osmotic pressure curves is also true for the curves for the other physical properties of gelatin. Thus Fig. 9 gives the curves for viscosity of 1 per cent solutions of gelatin bromide, oxalate, and tartrate, showing that they are practically identical when plotted with pH as abscissæ. Fig. 10 gives the curves

for swelling for gelatin oxalate, and chloride, which are also approximately identical.[5] The fact that the curves for viscosity and swelling are similar to those for osmotic pressure has been pointed out so often in previous papers that we need not dwell further on this point.

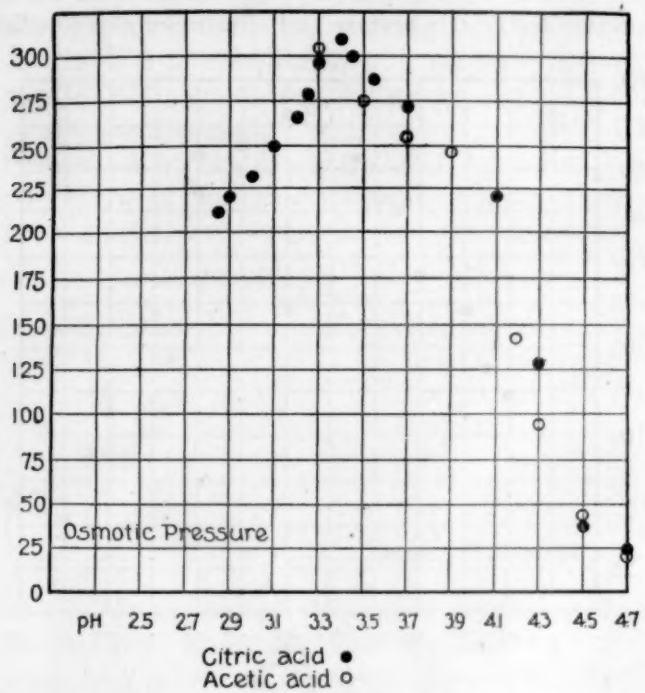

FIG. 8. Showing identity of influence of citric and acetic acids upon osmotic pressure of gelatin solution for the same pH. Curves in Fig. 8 are identical with those in Fig. 7.

[5] The close proximity of the effects of different acids does not harmonize with the contention of the colloid chemists who report a typical difference in the effects of the acids according to the nature of the anion. This is an error due to the fact that they failed to determine the pH of their solutions and to compare the behavior of gelatin solutions of the same pH, comparing instead the effects of equimolecular concentrations of different acids, without making any of the necessary corrections discussed in this paper.

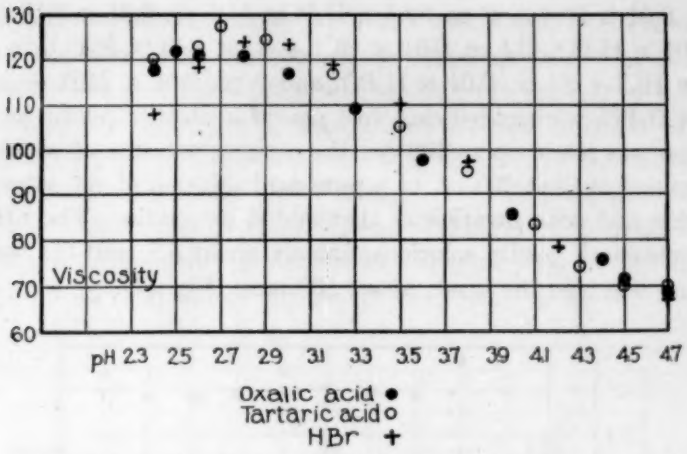

FIG. 9. Viscosity curves of gelatin treated with HBr, tartaric, and oxalic acids are identical when plotted over pH as abscissæ.

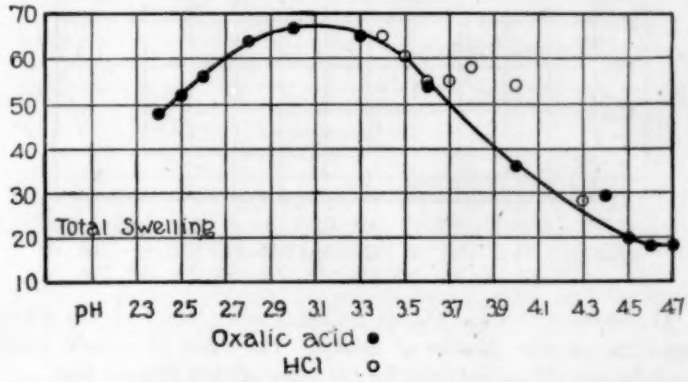

FIG. 10. Curves showing the influence of HCl and oxalic acid upon swelling of gelatin. The curves are identical when plotted over pH.

Antagonism Experiments between HCl and Phosphoric and Oxalic Acids.

It was to be expected that when H_3PO_4 or oxalic acid is added to HCl or HBr it can produce no or only a slight antagonistic effect. The results of actual experiments support this expectation. The same experiment as that with HCl and H_2SO_4 (represented in Fig. 4) was made by mixing different proportions of HCl and H_3PO_4.

15 cc. 0.01 N HCl + 0 cc. 0.01 N H_3PO_4; 13.5 cc. 0.01 N HCl + 4.0 cc. 0.01 N H_3PO_4; 12 cc. 0.01 N HCl + 8 cc. 0.01 N H_3PO_4 to 3 cc. 0.01 N HCl + 32 cc. 0.01 N H_3PO_4 and 0 cc. 0.01 N HCl + 40 cc. 0.01 N H_3PO_4 were added each to 1 gm. of isoelectric gelatin and the solution was made up to 100 cc., the osmotic pressure of which was determined against 400 cc. of a pure acid solution of the same constitution and concentration as that added to gelatin. The pH was the same in all gelatin solutions, namely about 3.5, and the osmotic pressure was also the same, about 300 mm. (Fig. 11).

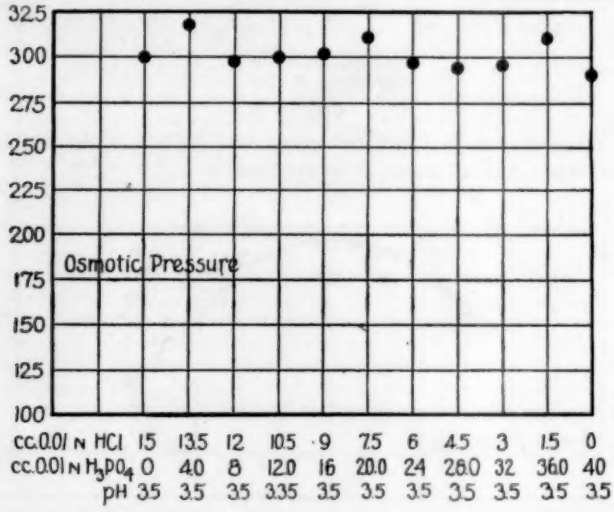

FIG. 11. Showing that H_3PO_4 has no antagonistic effect on the influence of HCl upon the osmotic pressure of gelatin. The curve of osmotic pressure of different mixtures of the two acids for the same pH is a straight line.

There is a slight antagonistic effect when we mix HCl and oxalic acid (Fig. 12). This is due to the fact that the amount of oxalic acid in combination with a given mass of gelatin is less than twice the amount of HCl (or HBr) in combination with the same mass of gelatin. It is possible that a small fraction of the oxalic acid acts like a dibasic acid on gelatin while the greater part acts like a monobasic acid.

The fact that the dibasic and tribasic anions which have no or only a slight antagonistic effect act like monobasic acids towards gelatin harmonizes with the hypothesis of aggregation. Our idea of aggregation is a stoichiometrical one, making the number of gelatin ions forming one aggregate of gelatin ions a simple multiple of the valency number of the polyvalent ion with which they are in combination. Since phosphoric, citric, tartaric, succinic, and practically also oxalic acids behave like monobasic acids, *i.e.* since they can bind only one gelatin molecule, they cannot be expected to cause any aggregate formation and hence cannot produce any antagonistic salt action.

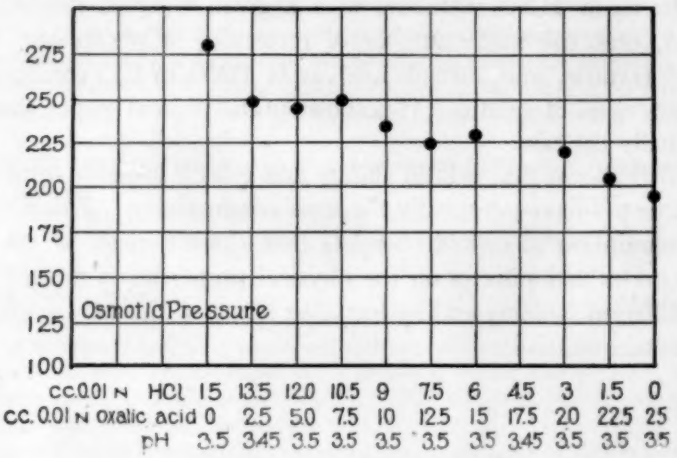

FIG. 12. Showing that oxalic acid has practically no antagonistic effect on the influence of HCl upon the osmotic pressure of gelatin.

SUMMARY.

1. When we plot the values of osmotic pressure, swelling, and viscosity of gelatin solutions as ordinates over the pH as abscissæ, practically identical curves are obtained for the effect of monobasic acids (HCl, HBr, HNO_3, and acetic acid) on these properties.

2. The curves obtained for the effect of H_2SO_4 on gelatin are much lower than those obtained for the effect of monobasic acids, the ratio of maximal osmotic pressures of a 1 per cent solution of gelatin sulfate

and gelatin bromide being about 3:8. The same ratio had been found for the ratio of maximal osmotic pressures of calcium and sodium gelatinate.

3. The curves representing the influence of other dibasic and tribasic acids, *viz.* oxalic, tartaric, succinic, citric, and phosphoric, upon gelatin are almost identical with those representing the effect of monobasic acids.

4. The facts mentioned under (2) and (3) permit us to decide between a purely chemical and a colloidal explanation of the influence of acids on the physical properties of gelatin. In the former case we should be able to prove, first, that twice as many molecules of HBr as of H_2SO_4 combine with a given mass of gelatin; and, second, that the same number of molecules of phosphoric, citric, oxalic, tartaric, and succinic acids as of HNO_3 or HCl combine with the same mass of gelatin. It is shown in the present paper that this is actually the case.

5. It is shown that gelatin sulfate and gelatin bromide solutions of the same pH have practically the same conductivity. This disproves the assumption of colloid chemists that the difference in the effect of bromides and sulfates on the physical properties of gelatin is due to a different ionizing and hydratating effect of the two acids upon the protein molecule.

STATEMENT OF THE OWNERSHIP, MANAGEMENT, CIRCULATION, ETC., REQUIRED BY THE ACT OF CONGRESS OF AUGUST 24, 1912,

OF THE JOURNAL OF GENERAL PHYSIOLOGY published bimonthly at Baltimore, Maryland, for April, 1919.

STATE OF NEW YORK } ss.
CITY OF NEW YORK }

Before me, a Notary Public in and for the State and county aforesaid, personally appeared Frank A. Dickey, who, having been duly sworn according to law, deposes and says that he is the Business Manager of THE JOURNAL OF GENERAL PHYSIOLOGY and that the following is, to the best of his knowledge and belief, a true statement of the ownership, management (and if a daily paper, the circulation), etc., of the aforesaid publication for the date shown in the above caption, required by the Act of August 24, 1912, embodied in section 443, Postal Laws and Regulations, printed on the reverse of this form, to wit:

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